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DEDICATION

The Editorial Committee of the conference on "Plasmids in Bacteria" dedicates this volume to Dr. I. C. Gunsalus in recognition and appreciation of his distinguished contributions to the fields of biochemistry and bacteriology. Gunny's independent scientific career spans some four decades and includes a number of innovative and fundamental contributions to our understanding of basic biochemical processes and bacterial metabolism. His early biochemical studies led to the isolation of the coenzymes pyridoxal phosphate and the discovery of lipoic acid. His later classical studies on the Pseudomonads provided the foundation for both fundamental and applied studies on the genetics and biochemistry of alkane and

primase genes since $IncI_1$ and R16 DNA primases are antigenically distinct. A survey (Ref. 15; Tab. 1) of a number of these plasmids showed that R64, R144, R621a, R864a, and RIP72 all contain nucleotide sequences homologous with the cloned ColIb-P9 sog gene. However, TP125, R724, pLG101 (IncB), R805, and R387 showed sequence homology with the R16 pri determinant but not with sog.

It is likely that many genetically different conjugation systems exist among plasmids of gram-negative bacteria (44) and since sog is apparently a transfer gene, it is possible that plasmids containing sog-like sequences specify the same system. If so, such plasmids should encode similar pili, which are required for cellular interactions in conjugation, and mutually interchangeable proteins for DNA transfer itself. Members of the I_1 complex can be arranged into different sets using pilus-specific phages Iα and PR64FS; the former adsorbs to the shafts of pili encoded by $IncI_1$ plasmids, R144 and R864a ($IncI_1$+B), and R621a (IncB), whereas PR64FS has a wider range since it also recognizes the tips of pili specified by R805a ($IncI_1$+B), and R387 (IncK) (Ref. 12; D.E. Bradley, pers. comm.). Hence, plasmids determining sensitivity to phage Iα possess sog-like sequences but the converse does not hold, as shown by RIP72.

At least 2 different DNA transfer mechanisms are recognizable among I_1 complex plasmids through the ability of representative plasmids to mobilize a small recombinant plasmid (pLG2009) carrying the origin of transfer (oriT) of ColIb-P9. Table 1 shows that $IncI_1$ plasmids, R144, R864a, and R621a mobilized pLG2009 with high efficiency. Thus these plasmids determine functionally similar DNA

Tab. 1. Mobilization of a recombinant plasmid carrying oriT of ColIb-P9 by conjugative plasmids of the I_1 complex.

Conjugative plasmid	Inc group	Primase gene type	Mobilization of pLG2009 [a]
ColIb-P9*drd-1* [b]	I_1	*sog*	82
R64*drd-11*	I_1	*sog*	513
R144*drd-3*	I_1+B	*sog*	50
R864a*drd-1*	I_1+B	*sog*	128
R621a	B	*sog*	231
RIP72	B	*sog*	0.12
TP125	B	*pri*	0.16
R724	B	*pri*	0.24
R805a	I_1+B	*pri*	0.078
R387	K	*pri*	2.5

[a] pLG2009 is a derivative of the nonmobilizable vector plasmid pED825 (2.2 kb; Ref. 17) containing *oriT* of ColIb-P9*drd-1* on a 0.8 kb fragment. Values show yield of pLG2009-containing transconjugants expressed as a percentage of transconjugants harboring the conjugative plasmid. Donor strains were mutant at *recA*.

[b] pLG221 (ColIb-P9*drd-1 cib*::Tn*5*) was used to allow selection for kanamycin-resistance.

transfer systems; they also contain sog-like sequences and comprise the set determining sensitivity to the RNA phage Iα. The other plasmids in Tab. 1 mobilized pLG2009 with frequencies of at least 30-fold lower than ColIb-P9, indicating that they specify DNA transfer mechanisms differing from that of the $IncI_1$ plasmid. With the exception of RIP72, this second group contains sequences homologous with the R16 pri gene. Thus, this survey indicates that plasmids with sog-like sequences specify the same or closely related conjugation systems, but the association is not absolute.

The RP4 pri gene (3.2 kb) is located between coordinates 40.3 and 43.5 in the recent SphI map of the plasmid (27), and the locus specifies 2 antigenically related polypeptides of 118 and 80 kd. The putative promoter is about 5 kb upstream of the beginning of the gene. Results of deletion mapping, together with immunological cross-reaction of proteins, indicates that the 2 polypeptides are separate in-phase translation products. Unlike sog products, both the 118 and 80 kd pri polypeptides possess DNA primase activity and this is located in a region towards the C-terminus of the products (26,28).

ROLE OF $IncI_1$ PLASMID PRIMASE IN CONJUGATION

Sog^- Mutants and Conjugative DNA Synthesis

We recently reported the isolation of a Sog^- $IncI_1$ plasmid called pLG250 and its use to identify the physiological role of the DNA primase (11). This mutant is a derivative of pLG221, which is ColIb-P9drd-1 conveniently marked by the presence of Tn5 in the colicin Ib gene, and it contains a hydroxylamine-induced mutation(s) that eliminates primase activity without affecting the apparent molecular masses of the sog polypeptides. Plasmid pLG250 was stably maintained in E. coli, implying that sog primase is not required for vegetative replication of the plasmid. Presumably the primers necessary for this process are generated by bacterial primase. However, the mutant was approximately 80% deficient in generating transconjugants in E. coli and Salmonella typhimurium matings, suggesting that the enzyme functions during conjugative synthesis of plasmid DNA. Consistent with this are the observations that sog is coordinately expressed with transfer genes, as judged by the effect of drd mutations in derepressing expression of these genes (41), and that sog activity and transfer proficiency were reduced by insertion of Tn5 in the E-2 fragment of ColIb-P9drd-1 (unpub. data).

During conjugation, a specific $IncI_1$ plasmid strand is transferred to the recipient cell (40) where the complementary strand is synthesized by DNA polymerase III holoenzyme in a process independent of RNA polymerase and E. coli primase (9,43). Involvement of sog primase in this process was investigated at the molecular level by measuring conjugative DNA synthesis in recipient cells (RCDS).

This was detected by measuring [^{14}C]thymine incorporation when thymidine kinase-deficient (tdk) donors were mated with thymine-requiring recipients under conditions that prevented the latter cells from replicating the resident DNA. This inhibition was achieved by exposing the cells to a large dose of ultraviolet light before mating, thereby inducing sufficient pyrimidine dimers to render the DNA unusable as a template for DNA synthesis (11).

Figure 2 shows the amount of RCDS detected when pLG221 (Sog^+) and pLG250 (Sog^-) donors were mated with irradiated dnaG3 recipients that were treated with rifampin to inhibit transcription of transferred DNA and thus to prevent synthesis of sog primase in transconjugant cells. Clearly RCDS in this mating system is dependent on plasmid primase which, in view of the rifampin treatment, is presumably supplied by the donor cell. The primary defect in the pLG250-mediated mating is not failure of DNA transfer itself, because the deficiency of RCDS was alleviated partially when the recipient cells harbored the pBR325-sog^+ recombinant pLG215 (Tab. 2) and thus contained plasmid primase before mating. We therefore infer that sog

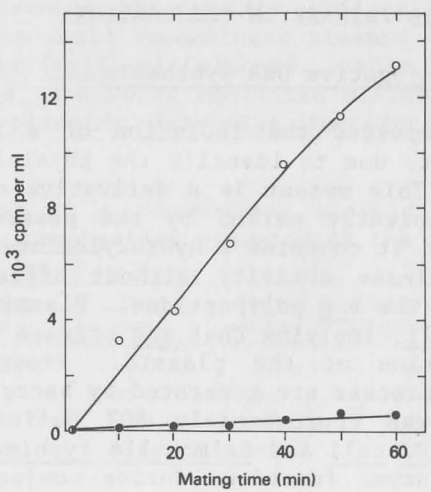

Fig. 2. Conjugative DNA synthesis in UV-irradiated, rifampin-treated dnaG3 recipients. Donor strain BW96 (dna^+ tdk rpoB) containing pLG221 (o) or the Sog^- derivative pLG250 (•) was mated at 41°C with recipient strain BW86 (dnaG3 uvrB thyA), UV-irradiated with 400 J/m^2 before use. Mating was in the presence of rifampin (100 µg/ml) and [^{14}C]thymine (63 mCi/mmol); TCA-precipitable radioactivity was determined at the times indicated. Further details are given elsewhere (11). Data have been corrected by subtraction of counts incorporated by unmated bacteria (maximum of 400 cpm per ml).

Tab. 2. Contribution of dnaG and sog primases to conjugative DNA synthesis in recipient cells.

Strains		Relative amount
Donor	Recipient	of RCDS
dna$^+$(pLG221)	*dnaG3*	1.0
dna$^+$(pLG250)	*dnaG3*	0.05
dna$^+$(pLG250)	*dna*$^+$	0.15
dna$^+$(pLG250)	*dnaG3*(pLG215)	0.37
dna$^+$(pLG250,pLG215)	*dnaG3*	0.64

BW96 was donor strain. BW86 and the homogenic *dna*$^+$ strain BW89 were recipients. pLG221 (Sog^+) and pLG250 (Sog^-) are IncIα plasmids while pLG215 is a pBR325-*sog*$^+$ recombinant. Matings were as for Fig. 2. Values are expressed as the amount of RCDS detected at 60 min relative to that when BW96(pLG221) was mated with BW86 (line 1).

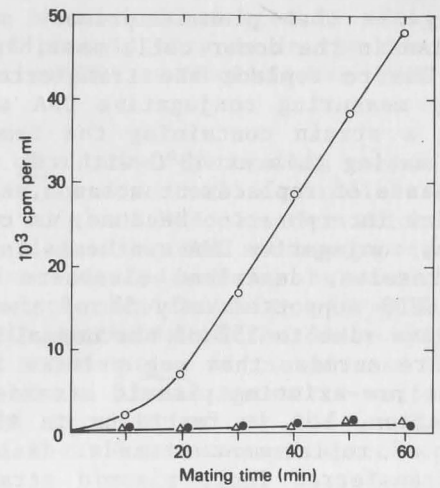

Fig. 3. Conjugative DNA synthesis in unirradiated, rifampin-treated dnaG3 recipients. Donor strain BW96 containing pLG221 (o) or pLG250 (•) was mated with BW86 recipients at 41°C. Counts incorporated when BW96(pLG221) was mated with BW86(pLG252) are also shown (Δ). pLG252 is a pBR325-eex^+ recombinant that excludes entry of pLG221. Other details are as for Fig. 2.

primase of the donor generates primers that are used after DNA transfer to initiate complementary strand-synthesis in the recipient; in a normal mating, primers might be made in the donor cell on the strand destined for transfer to be transmitted in a complex with the DNA, or they might be synthesized in the recipient cell following DNA and enzyme transfer.

The donor strain used in these experiments was *dna*$^+$ and it is apparent from Fig. 2 that *dnaG* protein of donor cells cannot substitute for *sog* primase in RCDS. However, *dnaG* protein supplied by recipient cells can utilize the transferred DNA but with lower efficiency than *sog* primase (Tab. 2). This substitution of bacterial for plasmid enzyme presumably explains the partial transfer-proficiency of pLG250 in genetic experiments.

Further evidence that primers for *sog*-dependent RCDS are made by enzyme supplied by the donor cell is the observation that pLG250 was complemented by the presence of pLG215 (Sog$^+$) in the donor cell (Tab. 1). Since pLG215 is nonmobilizable, cells harboring pLG250 and pLG215 transmit a functional *sog* gene to recipients with only low frequency.

It is significant that pLG215 complemented pLG250 more effectively if present in the donor rather than recipient cell (Tab. 2). This difference suggests that plasmid primase acts positively in plasmid DNA metabolism in the donor cell, possibly to promote efficient synthesis of DNA to replace the transferred plasmid strand. We examined this by measuring conjugative DNA synthesis in donor cells (DCDS), using a strain containing the temperature-sensitive *dnaB70* mutation and mating this at 43°C with *tdk* recipients. DCDS, which reflects synthesis of replacement strands, can be monitored in this system by thymine incorporation because, in contrast to vegetative DNA replication, conjugative DNA synthesis is a *dnaB*- independent process. The results, described elsewhere (11), showed that *dnaB70* donors of pLG250 supported only 5% of the normal amount of DCDS although they gave rise to 35% of the normal yield of transconjugants. We therefore surmise that *sog* primase is inessential for transfer of at least pre-existing plasmid strands, as indicated by transconjugant formation, but it functions in the donor to allow efficient synthesis of replacement strands. Assuming, by analogy with F, that the transferred IncI$_1$ plasmid strand is transmitted with its 5' terminus leading, RNA primer would be required for DCDS if the 3' terminus were unavailable for extension because of its association with, for example, some component of the cell membrane (44). Alternatively, DCDS may be a discontinuous process needing multiple priming events.

In summary, we suggest that IncI$_1$ plasmid primase plays 2 roles in conjugation: it acts in the donor cell to promote synthesis of DNA to replace the transferred plasmid strand, and it generates primers that are utilized for complementary strand-synthesis in the recipient. This contrasts models in which DCDS of R64*drd-11* requires primers generated by RNA polymerase, with RNA synthesis initiating DNA transfer itself (13). These models stemmed from the observation that transfer and DCDS were more sensitive to rifampin than to chloramphenicol during mating of *dnaB* donors and minicells at 42°C (19). However, in another study (7), rifampin did not block early transfer of ColIb-P9*drd-1* between *dna*$^+$ bacteria at 37°C and, since the antibiotic was found to cause a general disruption of DNA metabolism in bacteria harboring IncI$_1$ *drd* plasmids it was suggested that this effect of rifampin-treatment was responsible for curtailment of DNA transfer.

The more recent finding that the yield of R64*drd-11* transconjugants was reduced by about 90% when cytidine-utilization was prevented in *dnaB* donors provides alternative, although indirect, evidence that transfer requires RNA synthesis in the donor cell (30). The enzyme responsible might be IncI$_1$ plasmid primase, since this generates primers initiated with cytidine, but it is stressed that our studies with pLG250 do not indicate requirement of this enzyme for transfer of pre-existing ColIb-P9 DNA. Clearly more remains to be learned about priming of DCDS and its relationship to DNA transfer. Further progress is likely to require better understanding of the nature of the 3' teminus created by nicking at *oriT*, and of enzymes that unwind plasmid strands in the donor cell. The latter aspect of conjugative DNA metabolism has been reviewed elsewhere (44).

Participation of a primer-generating enzyme in synthesis of replacement strands in the donor cell contradicts a central feature of the rolling circle model of DNA transfer which predicts continuous elongation of the plasmid strand undergoing transfer (20). Other contradictory evidence includes delay between first-round transfer of R64*drd-11* and second-round transfer of replacement DNA (18), requirement of protein synthesis in the donor cell for each round of transfer (19), and accumulation of transferred ColIb-P9*drd-1* DNA in the form of circular and linear monomeric units, even under conditions that impeded termination of the conjugation process and allowed transfer of about 5 genome equivalents to each cell (8).

Transfer of Plasmid DNA Primase to Recipients

Participation of *sog* primase of donor cells in RCDS suggests that the enzyme may be transmitted between conjugating cells. Since the primase can utilize a variety of DNA templates for primer synthesis, including the bacterial chromosome of *dnaG* mutants, 1 functional test for primase transfer asks whether unirradiated *dnaG3* recipient cells recover ability to synthesize chromosomal DNA when mated

with donors that are the sole source of the enzyme. This test assumes that RNA primers function on the template that serves for their synthesis, and that if RNA primers were transmitted during conjugation, they would be hydrogen-bonded to plasmid DNA and thus unable to act in trans to promote replication of bacterial DNA native to the recipient cell.

Comparison of the data in Fig. 2 and 3 shows that 3.6-fold more RCDS was detected in rifampin-treated dnaG3 recipients of pLG221 when the native DNA was unirradiated rather than rendered defective as a template for DNA synthesis by irradiation. Variation in the amount of DNA transfer in the 2 matings does not explain the difference, because irradiation of recipients only marginally reduced the amount of DNA transferred from this pedigree of donor cells. On the basis that incorporation of 1,200 counts per min per ml in this experimental system reflects synthesis of 31 x 10^6 daltons of single-stranded DNA (9), and that pLG221 (ColIb-P9::Tn5) has a molecular weight of about 69 x10^6, it is estimated that the average recipient in Fig. 3 supported synthesis of DNA equivalent to 36 single strands of pLG221 in 60 min. This high value itself suggests that the dnaG recipients were recovering their ability to replicate DNA during mating.

Recovery of bacterial DNA synthesis in mated dnaG3 recipient cells was confirmed directly by mating pLG221 donors with unirradiated, rifampin-treated, dnaG3 recipients for 60 min, as in Fig. 3, and then measuring the proportions of labeled DNA that were homologous with the plasmid and bacterial chromosome. This approach showed that 69% of the DNA labeled in the mated dnaG recipients was homologous with bacterial DNA and 24% with pLG221. Since the method showed that E. coli DNA and pLG221 share only about 2% homology, these results prove that the dnaG3 recipient cells recovered the ability to synthesize chromosomal DNA during mating, synthesizing almost 3 times more bacterial than plasmid DNA.

This recovery was dependent on plasmid DNA primase because few counts were incorporated if the donor strain harbored pLG250 (Sog^-) rather than pLG221 (Fig. 3). Furthermore, it was presumably mediated by sog protein derived from donor cells, because the recipient cells were treated with rifampin throughout mating to inhibit gene expression in plasmid transconjugants. The possibility that this treatment did not fully inhibit RNA polymerase native to the recipient cell, or that antibiotic-resistant RNA polymerase was transmitted from the donor cells, is refuted by the observation that rifampin-treated recipients also recovered the ability to synthesize bacterial DNA when mated with donors harboring both pLG250 and the small Sog^+ recombinant plasmid pLG215 (data not shown). Such Sog^+ cells contain functional plasmid primase but cannot donate a sog^+ gene during conjugation with significant frequency because pLG215 is not mobilizable.

These several results, to be described in detail elsewhere, lead to the inference that sog primase is transferred from donor to recipient during mating. The alternative explanation, that the donors transmit multiple mobile RNA primers that can function in trans to prime bacterial DNA synthesis in the recipient cell, is inconsistent with the current concept that a short RNA primer promotes DNA synthesis on the template that served for its own formation.

Mode of Transfer of Sog Primase

The phenomenon of entry exclusion has been exploited to determine whether sog primase is transferred between cells by a conjugation-dependent process. Entry exclusion is a barrier that prevents DNA transfer between cells harboring the same or a closely related plasmid. In F-mediated conjugation, eex genes may operate at 2 different stages: to reduce the formation of stable mating aggregates and to prevent DNA transfer between those aggregates that are formed (1). An entry exclusion gene(s) of ColIb-P9 has been cloned in pBR325: the recombinant plasmid is designated pLG252 and it contains a 3.5 kb EcoRI-generated fragment of the $IncI_1$ plasmid, which is E-9 according to the numbering system of Uemura and Mizobuchi (38). When present in a recipient strain, pLG252 decreased transfer of pLG221 by more than 98%, as measured by yield of transconjugants and by transmission of radioactively labeled DNA. The ColIb-P9 fragment present in pLG252 resembles the exclusion-determining fragment of R144 (21), both with regard to conferred phenotype and in containing a 1.15 kb EcoRI-HindIII fragment adjacent to a 0.51 kb HindIII-SalI fragment. Since the cloned R144 eex gene(s) was shown to prevent conjugation at a stage subsequent to the formation of mating aggregates, we postulate that the corresponding gene in pLG252 functions likewise, possibly to inhibit DNA transfer between aggregated cells.

Figure 3 shows that donors of the Sog^+ conjugative plasmid pLG221 were unable to promote the rescue of bacterial DNA synthesis in dnaG recipient cells when the latter contained pLG252. This implies that sog primase is transmitted between mating cells by a conjugation-dependent process and, since the enzyme binds to single-stranded DNA, as revealed by its purification properties (29), we suggest that it is transmitted in a complex with the transferred plasmid strand. While it is simpler to assume that primers for RCDS are made following protein and DNA transfer, it is possible that they are generated in the donor cell and transmitted to the recipient bonded to the transferred DNA. If primers synthesized in vivo were as short as those made in vitro, they would require stabilization on the transferred strand and plasmid primase might fulfill this role, thus explaining the need for co-transfer of primers and enzyme.

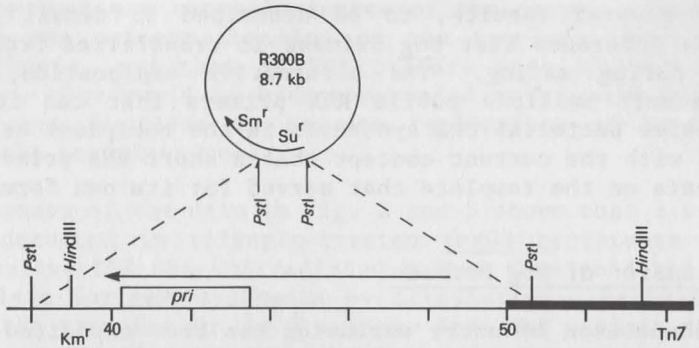

Fig. 4. Construction of pLG261: an R300B derivative carrying the pri region of the IncP plasmid pRP26. The latter is a RP4::Tn7 mutant specifying overproduction of plasmid primase (26). Map coordinates of RP4, location of pri and the putative transcript (line with arrowhead) are taken from Lanka et al. (27,28). Details of the Tn7 region are unpublished data of P.T. Barth and K. Ellis. Plasmid pRP1 is RP4 containing Tn7 inserted at about coordinate 41 (26,27).

ROLE OF RP4 PLASMID PRIMASE

The role of this enzyme was first studied by Lanka and Barth (26) using RP4 mutants carrying Tn7 insertions that inactivated primase activity. The insertions map within the operon that contains pri and the location of 1 (pRP1) is defined in the legend to Fig. 4. These mutants were lost from E. coli and S. typhimurium cells with a frequency of about 3% during 20 generations of unselected growth, indicating that the DNA primase plays a minor role in the maintenance of RP4. However, the requirement appears to be for resolution of recombination intermediates rather than for priming of vegetative replication itself (4).

Compared with RP4, E. coli donors of the Pri$^-$ mutants were defective in conjugation in some interspecific matings (26). Normal yields of transconjugants were detected when 6 bacterial species, including E. coli and Psuedomonas spp., were recipient strains but a reduced yield was recorded in 5 other matings, the decrease being about 25-fold in the case of S. typhimurium or Proteus mirabilis recipients. Back transfer from S. typhimurium donors established that the variation depended on the recipient rather than the donor strain. Assuming that a single strand of RP4 is transferred during conjugation, it was suggested that the plasmid primase acts in conjugation to generate primers for complementary strand-synthesis in the recipient cell, and that this activity is necessary for transconjugation formation in those species, such as S. typhimurium, that produce a primase which does not efficiently recognize the transferred DNA (26).

In collaboration with Dr. P.T. Barth, we have examined further the role of RP4 primase in conjugation, using pRP1 as a representative Pri$^-$ plasmid (26) and pRP2 as a control. The latter plasmid contains a Tn7 insertion near the RP4 tetracycline-resistance gene, which is outside the known transfer genes (5). To allow introduction of the pri$^+$ gene into different recipient species, the gene was cloned in R300B, giving pLG261 (Fig. 4). R300B is a broad host-range plasmid of the IncQ group that is indistinguishable from RSF1010, R1162, and NTP2 (6).

Table 3 shows the relative yields of transconjugants formed when E. coli donors were mated with E. coli or S. typhimurium LT2 recipients. Transconjugant formation by the Pri$^-$ plasmid was reduced by about 95% in the interspecific mating, in accordance with previous data (26), but it was also diminished, by about 30%, when E. coli recipients were used. Since these deficiencies were partially rectified when the recipient strains contained the Pri$^+$ recombinant plasmid pLG261, it is apparent that RP4 primase is inessential for DNA transfer itself, but its products of reaction are needed in the recipient cell, presumably to initiate efficient conjugative DNA synthesis on transferred DNA. Clearly, dnaG protein of donor cells cannot bypass this requirement for RP4 primase, and even primer-generating enzymes specified by E. coli recipients are unable to substitute completely.

Tab. 3. Relative yield of transconjugants generated by RP4 Pri$^+$ and Pri$^-$ plasmids.

Recipient strain	Transconjugants
E.coli K-12	
C600	69
C600(pLG261)	98
S.typhimurium LT2	
SL329	4
SL329(pLG261)	61

Values show the yield of kanamycin-resistant, thymine non requiring transconjugants generated by W3110T$^-$(pRP1) donors, expressed as a percentage of that obtained with the W3110T$^-$(pRP2) control strain. Mating occurred on nitrocellulose filters placed on nonselective medium at 37°C for 3 hours. The construction of pLG261 is summarized in the legend of Fig. 4.

CONCLUDING REMARKS

A variety of conjugative plasmids encode DNA primases, the best studied being those of ColIb-P9 and RP4. These plasmids specify different but enzymatically similar primases that act in conjugative DNA metabolism. The ColIb-P9 enzyme is inferred to promote synthesis of DNA in the donor cell to replace the transferred strand, and it is apparently transmitted by a conjugation-dependent process to the recipient where its products initiate complementary strand-synthesis on the transferred DNA. The role of RP4 primase has not been studied at the molecular level, but genetic data suggest a function analogous to that of the ColIb-P9 enzyme.

While these primases are needed for efficient conjugative DNA synthesis in the recipient cell, they are apparently redundant for plasmid replication. Presumably primers for the latter process are generated by bacterial enzymes, but these are unable to substitute fully for plasmid primases during conjugation. This may be explained if DNA replication is unidirectional, with only 1 strand supporting synthesis of multiple primers for discontinuous DNA synthesis. The second strand might be deficient in recognition sites for bacterial primer-generating enzymes, since it acts as template for leading strand synthesis. If this second strand were transferred, efficient complementary strand-synthesis in the recipient might require a conjugation-specific priming mechanism and plasmid primase may contribute to 1 such process. RP4 replication is unidirectional and proceeds with opposite orientation to DNA transfer (2,31); if it is assumed that a distinct strand of RP4 is transferred with 5' terminus foremost, the transmitted strand will be the one that serves as template for leading strand synthesis. It is unknown whether the same applies to ColIb-P9 because the directions of replication and transfer of this plasmid have not been established.

Since plasmid primases render initiation of complementary strand-synthesis independent of bacterial primer-generating enzymes, they may contribute to host range by facilitating conversion of transferred DNA into double-stranded molecules in diverse bacterial species. The broad host-range of RP4 is well documented and, while ColIb-P9 is reported to be maintained in only a few enteric bacterial species (23) its conjugation system apparently allows DNA transfer between a much wider range of bacteria, as indicated by the ability of *E. coli* donors of pLG221 to denote Tn*5* to DNA of *P. putida*, *Methylophilus methylotrophus*, and *Alcaligenes eutrophus* (G. J. Boulnois et al., submitted for publication).

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PROMOTERS IN THE TRANSFER REGION OF PLASMID F

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INTRODUCTION

Regulation of the conjugation (*tra*) genes of IncF plasmids occurs by a complex, 2-stage process. Most IncF plasmids carry 2 fertility inhibition genes, *finO* and *finP*, the products of which act in concert to prevent transcription of *traJ*, a gene which has its own promoter (6,22). F itself lacks *finO* but carries both a *finP* gene and the site at which FinOP regulation occurs, *fisO*. In turn, the *traJ* protein (pTraJ) is required for transcription of the major 32-kb *traY→Z* operon (10,22). The transfer region contains 2 further promoters, that for *traM* (21,23), and a weak promoter that also transcribes *traI* and *traZ* (1,23). There have also been suggestions that the F *traT* surface exclusion gene has its own promoter (18), as does the *traT* gene of R100 (16), although the polarity on surface exclusion and *traD* of Mu insertions into the *traYZ* operon argues against this (8,10). These regulatory stages were reviewed by Willetts and Skurray (24).

We have constructed fusions of all the *tra* promoters, and of the *finP* promoter, to the *galK* gene using the pKO plasmids described by McKenney et al. (14) or other plasmids derived from these. Measurement of the galactokinase levels produced by these plasmids under induced or repressed conditions has given further information about the regulatory systems, and allowed the *tra* promoter strengths to be compared to each other and to other prokaryotic promoters. We have paid particular attention to the promoter for the *traYZ* operon

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since this is positively controlled, and such systems are relatively rare.

RESULTS AND DISCUSSION

Fusion of the tra Promoters to galK

In vitro recombination was used to fuse all of the potential tra promoters, and the putative finP promoter, to the galK gene. Vector plasmids were pKO1 and pKO4 (14), pHR11 (15), and 2 derivative plasmids constructed in our laboratory, pED101 and pED115. The latter contain the cloning sites BamI HindIII ClaI EcoRI and EcoRI ClaI HindIII BamI prior to galK, respectively, and are particularly useful since TaqI or HpaII/MspI termini can be readily cloned into ClaI termini by virtue of their CG single-strand extensions.

A variety of previously cloned segments of the F transfer region served as sources of the promoter fragments. Those fragments that were cloned into pKO-derived vectors are shown in Fig. 1. Details of the constructions will be presented elsewhere (P. Mullineaux and N. Willetts, in preparation). The galactokinase levels determined by these plasmids were measured in the presence or absence of other plasmids providing finO, finP or traJ, as appropriate (Tab. 1).

Two plasmids containing traJ-galK fusions were constructed, one (pED106) with a SalI-BglII fragment, and the second (pED121) with a TaqI fragment that by comparison was missing the 81 bp between the TaqI site in the traJ leader mRNA and the BglII site in traJ (Fig. 2). Genetic complementation tests showed that pED106, but not pED121, carried the finP gene: a part of this gene must therefore be located in the 81-bp TaqI-BglII region. This location is consistent with the sequence mapping of 2 finP mutations in this region (Fig. 2), and suggests that finP is a small gene corresponding to the DNA strand complementary to that encoding the traJ leader mRNA.

The galactokinase level of pED106, but not of pED121, was reduced by the FinOP system (Tab. 1), indicating that all or part of the site of action, fisO, also lies in the 81-bp TaqI-BglII region. pED121 includes the entire striking 12-bp perfect inverted repeat sequence located immediately prior to the TaqI site (Fig. 2), but not the F DNA sequence immediately following this. We hypothesize by comparison with transcriptional termination sites (19), that the fisO site includes both the inverted repeat sequence and the following DNA sequence where the usual run of 4 to 8 T's is replaced by specific binding of the FinOP repressor. Regulation of traJ would therefore be by premature termination of transcription rather than by prevention of initiation. Confirmation of this proposed location for the fisO site awaits the sequencing of fisO mutations such as those derived by Finnegan and Willetts (4).

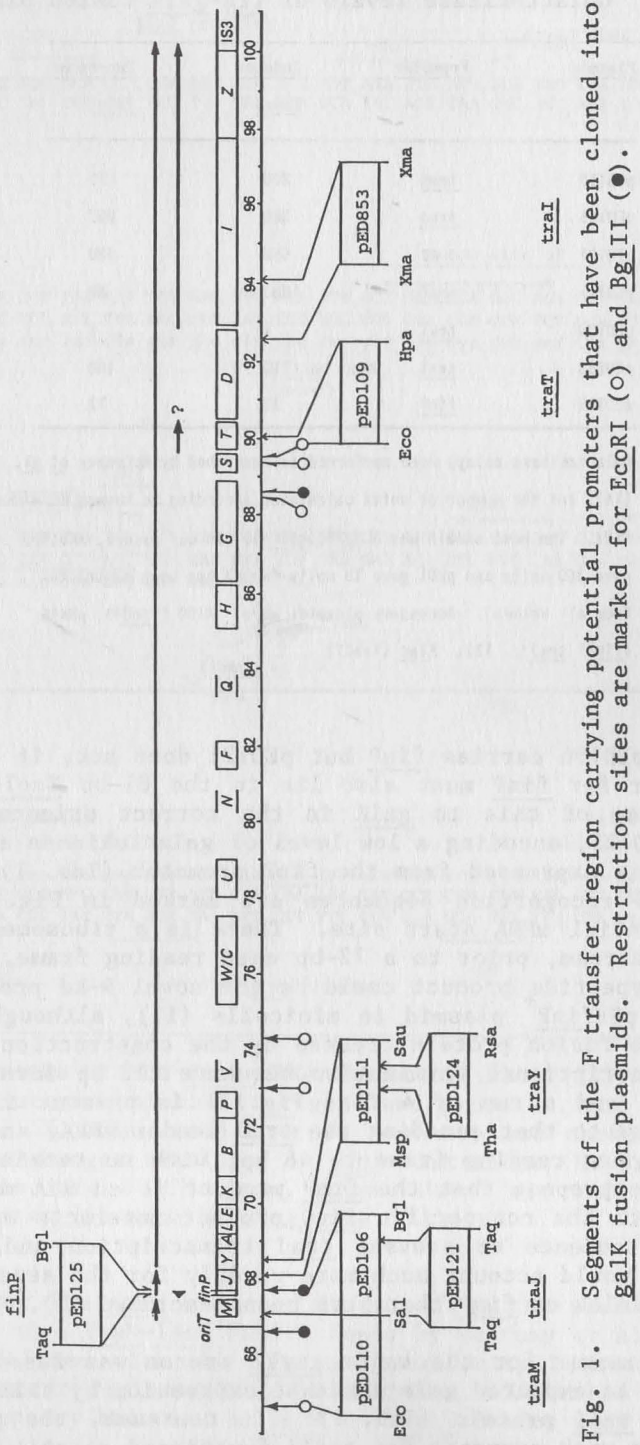


Fig. 1. Segments of the F transfer region carrying potential promoters that have been cloned into galK fusion plasmids. Restriction sites are marked for EcoRI (○) and BglII (●).

Tab. 1. Galactokinase levels of tra-galK fusion plasmids

Plasmid	Promoter	Induced level	Repressed level
pED110	traM	220	170
pED106	traJ	580	200
pED121	traJ	550	520
pED111	traY	1230	80
pED109	traT	1	1
pED853	traI	110	100
pED125	finP	12	12

Galactokinase assays were performed as described by McKenney et al. (14), and the number of units calculated according to Newman et al. (15). The host strain was JC3272 (galK-). In our assays, pKG1800 gave 360 units and pKO1 gave 18 units (which has been subtracted from all values). Accessory plasmids were: R100 (finO+), pNK46 (finP+ traJ-; 12); Flac (traJ+).

Since pED106 carries finP but pED121 does not, it follows that the promoter for finP must also lie in the 81-bp TaqI-BglII fragment. Fusion of this to galK in the correct orientation gave a plasmid, pED125, encoding a low level of galactokinase activity that we presume is expressed from the finP promoter (Tab. 1). Potential -35 and -10 recognition sequences are marked in Fig. 2, together with a potential mRNA start site. There is a ribosome binding sequence downstream, prior to a 72-bp open reading frame, and the potential polypeptide product could be the novel 4-kd protein synthesized by a pBRfinP+ plasmid in minicells (11), although this might instead be a fusion protein created by the construction (11,21). A strong transcriptional termination sequence (12 bp inverted repeat, 5 GC pairs, and a run of 4 T's; Fig. 2) is present in the strand complementary to that encoding the traJ leader mRNA, and this would reduce the open reading frame to 48 bp, with no termination codon. We therefore propose that the finP product is an RNA molecule that together with the nonspecific finO product interacts with its complementary sequence to prevent traJ transcription and/or translation. This would account much more readily for the several plasmid-specific alleles of finP that have been described (5).

The promoter for the major traYZ operon was fused to galK in pED111, and as expected galactokinase expression by this plasmid was induced by traJ protein (Tab. 1). In contrast, the promoter for traM and the weak promoter for traIZ functioned constitutively.

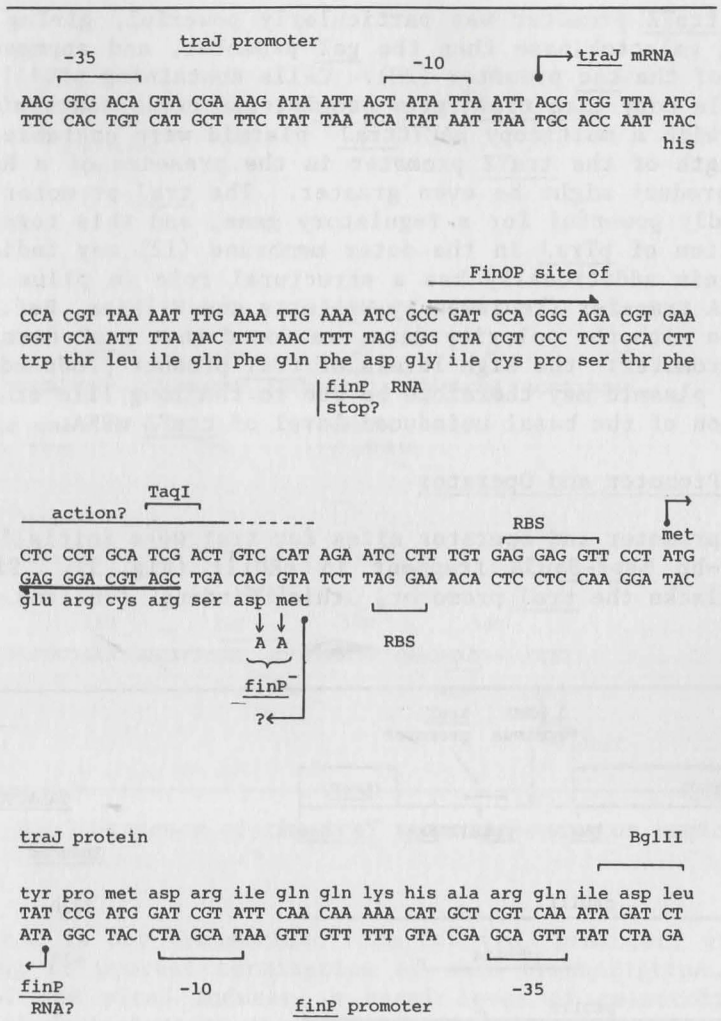


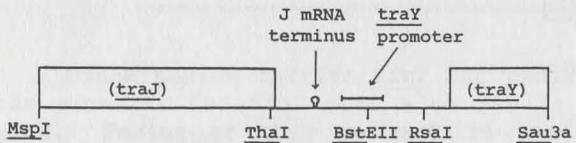
Fig. 2. Sequence of the traJ mRNA leader sequence (21). The likely location of the finP gene and promoter, and its potential 24 amino acid protein product, are shown. Two G→A base changes resulting in finP- mutations (N. Willetts, J. Maule, and R. Thompson, unpub. data) are indicated. RBS denotes ribosome binding site.

This result for the traM promoter differs from the pTraJ induction of expression of a traM-lacZ fusion found by Gaffney et al. (8) that might be correlated with the sequence similarity between the traM and traYZ promoter regions (Ref. 21; see Fig. 4 below). The reason for the discrepancy between the lacZ and galK fusion data remains to be elucidated.

The traYZ promoter was particularly powerful, giving about 4-fold more galactokinase than the gal promoter, and approaching the strength of the tac promoter (20). Cells containing pED111 together with single copy Flac traJ$^+$ grew poorly, and cells containing pED111 together with a multicopy pACYCtraJ$^+$ plasmid were unstable, so that the strength of the traYZ promoter in the presence of a high level of traJ product might be even greater. The traJ promoter was also unexpectedly powerful for a regulatory gene, and this together with the location of pTraJ in the outer membrane (12) may indicate that this protein additionally has a structural role in pilus formation and/or DNA transfer (reviewed by Willetts and Wilkins, Ref. 25). In accordance with the polarity data, we found that traT does not have its own promoter: the high levels of traT product produced by a repressed F plasmid may therefore be due to the long life or efficient translation of the basal uninduced level of traYZ mRNA.

The traY Promoter and Operator

The promoter and operator sites for traY were initially located on a 395-bp MspI-Sau3a fragment in pED111 (Fig. 3). Since this fragment lacks the traJ promoter, this finding confirms that the



	GALACTOKINASE	
	Induced	Uninduced
pED111	1230	80
pED124	630	140
pED119	8	7
pED114	100	100
pED118	1	1
pED116	130	90
pED117	1340	110

Fig. 3. The traJ-traY intercistronic region, showing segments fused to galK and the galactokinase levels produced by the resultant plasmids in the presence or absence of Flac traJ$^+$. The termination site for traJ transcription (7), and the potential traY promoter, are marked.

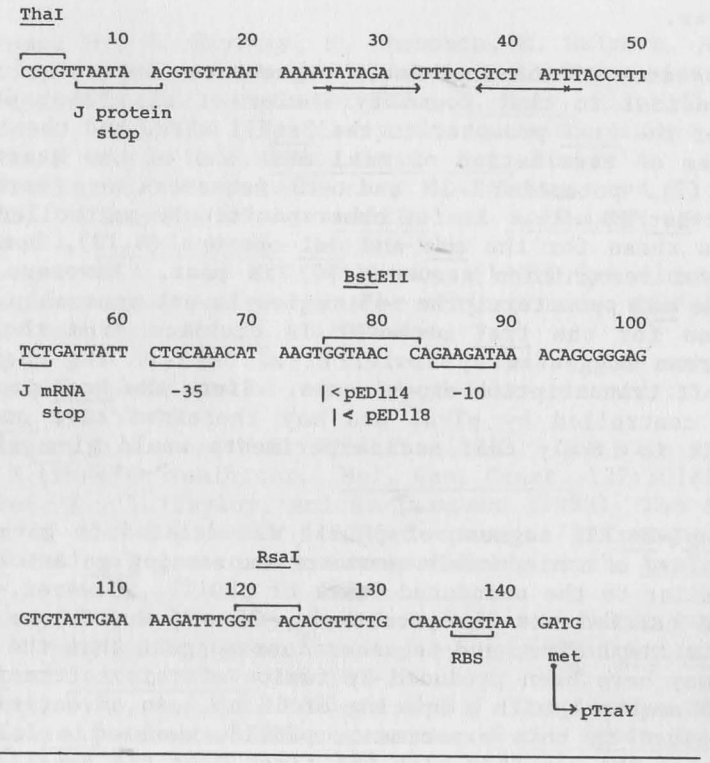


Fig. 4. Sequence of the traY promoter-operator region.

traYZ operon is not transcribed from the traJ promoter, with pTraJ functioning to prevent termination of such transcription. In the absence of the pTraJ inducer, a basal level of galactokinase was synthesized equivalent to about 6% of the induced level. This compares to <5% as measured by mRNA hybridization (22), and <0.3% measured in Flac traY-(MuAp) lac fusions (8): the reason for the higher level in the present case is unknown.

The promoter was located more accurately in 2 ways. Firstly, the central 118-bp ThaI-RsaI fragment was fused to galK in pED124, and this was shown to retain the inducible promoter (Fig. 3). Secondly, pED111 was digested with BstEII followed by brief BAL-31 treatment prior to religation, to give short deletion mutants. These are currently being sequenced, but polyacrylamide gel determination of the MspI-Sau3a fragment size showed that <20 bp had been deleted. One such plasmid was pED119, and this had totally lost ability to produce galactokinase. The traY promoter therefore covers or is very close to the BstEII site, and the uninduced level

of galactokinase produced by pED111 is most likely due to the same traY promoter.

The sequence of the ThaI-Sau3a segment was determined (Fig. 4) and is identical to that found by Fowler et al. (7). Given the proximity of the traY promoter to the BstEII site, and the locations of the sites of termination of traJ mRNA and of the start of the traY gene (7), potential -35 and -10 sequences are marked that bracket the BstEII site. As for other positively controlled promoters such as those for the ara and mal operons (3,13), homology to the consensus recognition sequence (9) is poor. However, in contrast to the mal promoters, the -45 region is not unusually GC-rich. Our location for the traY promoter is distinct from those about 50 bp upstream suggested by Fowler et al. (7) on the basis of in vitro run-off transcription experiments. Since the traY promoter is positively controlled by pTraJ and may therefore have an unusual sequence, it is likely that such experiments would give misleading results (17).

The MspI-BstEII segment of pED111 was deleted to give pED114, which contained a noninducible promoter expressing galactokinase at a level similar to the uninduced level of pED111. However, the vector plasmid carried the tet promoter's -35 sequence 10 bp upstream from the ClaI/MspI site, and sequence data suggest that the promoter in pED114 may have been produced by fusion of this to the traY promoter's -10 sequence with a spacing of 18 bp. An adventitious Gal mutant obtained in this experiment, pED118, carried a deletion of 7 bp of vector DNA together with the first G of the BstEII recognition sequence. It gave no galactokinase activity, confirming that a part of the traY promoter must lie to the left of the BstEII site, and that the galactokinase synthesized by pED114 is therefore probably not derived from an uninduced traY promoter.

A further deletion of the RsaI-Sau3a fragment of pED114 gave pED116, which also carried the putative fusion promoter. In an in vitro reconstruction experiment, the MspI-BstEII segment of pED111 was recombined with the BstEII-RsaI segment of pED116 to give pED117. This recovered inducibility by pTraJ, suggesting that the traY operator site is upstream of the BstEII site. More precise location of this site awaits the sequencing of mutations leading to pTraJ independence (2) or pTraJ noninducibility, or isolation of functional pTraJ from the outer cell membrane followed by "footprinting" experiments to locate its binding sequence.

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CONJUGATIVE SEX PLASMIDS OF STREPTOMYCES

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INTRODUCTION

Much is known about the ways in which the conjugative plasmids of gram-negative bacteria cause their hosts to mate (1). Detailed knowledge has been obtained about how the transfer of plasmid DNA occurs (2). The mechanisms by which such plasmids promote the mobilization of chromosomal genes have also been extensively studied (3-6). In contrast, very little is known about such phenomena for Streptomyces plasmids (7). However, now that many Steptomyces plasmids can be isolated with ease (8), genetically manipulated in vitro, and returned to the host cells by transformation of protoplasts (9,10) we can hope for more rapid progress in the understanding of the molecular biology of these plasmids. Here we review some facts and ideas about Streptomyces conjugation which may serve as a basis for future experimentation. As we see, conjugative Streptomyces plasmids are likely to possess properties interestingly different from those of plasmids of gram-negative bacteria and so they should repay a detailed investigation.

GENERAL FEATURES OF STREPTOMYCES PLASMIDS

The first conjugative plasmid to be identified in a gram-positive bacterium was SCP1 in the genetically studied Streptomyces coelicolor A3(2)(11). SCP1 was recognized through its influence on the recombination of host chromosomal genes ("fertility") and because it determines production of and resistance to a diffusible inhibitor of growth and sporulation, later identified as the antibiotic methylenomycin (12). Various derivatives of SCP1 have been characterized genetically, including those mediating high-frequency donation of chromosomal markers in which SCP1 seems to be stably in-

tegrated into the host chromosome, and autonomous SCP1-prime plasmids carrying segments of host DNA (13). Complete molecules of SCP1 DNA have not been isolated but segments of SCP1 have been cloned, including genes coding for methylenomycin resistance (14) and production (15); uncharacterized segments of the plasmid (15); and a region capable of autonomous replication when ligated with DNA carrying a selectable marker to form circular molecules (M.J. Bibb and J.M. Ward, pers. comm.). The size of SCP1 has been estimated to be in the range of 150 kb to at least 200 kb (15,16).

The first Streptomyces plasmid to be isolated physically was an initially cryptic CCC molecule of 31 kb (17) which was distinct from SCP1 and was later shown to be identical to a second sex plasmid, SCP2, of S. coelicolor. This plasmid had been identified through the occurrence of spontaneous variants (SCP2*) capable of promoting increased fertility in S. coelicolor A3(2)(18). The copy number of SCP2 in S. coelicolor is 1-4 per chromosome. This plasmid has not been shown to interact stably with the chromosome-indeed, an exhaustive search for high-frequency donors of chromosomal markers and for SCP2-prime plasmids was unsuccessful (J.A. Ewing, pers. comm.). However, the recent development of stable cloning vectors derived from SCP2* (19) has enabled segments of the host chromosome to be inserted into the plasmid, to provide artificial SCP2*-prime derivatives. Some of these, carrying segments of DNA which include genes for biosynthesis of the antibiotic actinorhodin (20), have been used in some genetic experiments described herein.

Many other plasmids have now been identified as CCC DNA (or occasionally as linear molecules; Ref. 21) in a wide variety of Streptomyces species (reviewed in Ref. 7). In contrast to SCP2 and (presumably) SCP1, several have a high copy number. Thus, pIJ101, a plasmid of 8.9 kb found naturally in S. lividans ISP5434, exists in 40-300 copies per chromosome depending on the age of the culture (22). A copy number as high as 800 has been reported for pNM100 in S. virginiae (23). A further interesting series of plasmids is represented by SLP1, pIJ110, and pIJ408. These are found as autonomous plasmids in S. lividans 66 after mating this strain with S. coelicolor A3(2), S. parvulus ATCC 12434, or S. glaucescens ETH 22794 respectively (24). They arise by the "looping out" of DNA sequences which occur as part of larger replicons (in the case of SLP1 in S. coelicolor this has been shown to be the chromosome; Ref. 25) and their conjugal transfer to S. lividans.

Most Streptomyces plasmids that have been studied give rise to "pocks" when a plasmid-carrying spore (or other plating unit) develops within a lawn of plasmid-free individuals. Pocks are circular areas, up to a few millimeters in diameter, in which the gross appearance of the culture is altered; usually the development of aerial mycelium and spores is delayed or prevented, and there may be precocious production of secondary metabolites, such as actinorhodin

and undecylprodigiosin in S. coelicolor and S. lividans. Pock-formation (also called the Ltz$^+$ phenotype; Ref. 25) is discussed in more detail below.

STREPTOMYCES PLASMIDS AS TRANSFERABLE FERTILITY FACTORS

Minimum Transfer Regions

Table 1 lists some examples of comparatively small plasmids of Streptomyces that have been shown to be capable of conjugal transfer. For 3 plasmids, the segment of the plasmid required for transfer and pock formation has been partially defined. This has not been done for the other 4 plasmids. However, assuming that the essential replication functions of the plasmids occupy at least 2 kb (Tab. 2), it is apparent that not more than 3-10 kb of DNA is required for transfer and pock-formation by any of the 7 plasmids (and the upper end of this range is probably a considerable overestimate). This contrasts with the 15-35 kb transfer regions of several plasmids that have been analyzed in gram-negative bacteria (26-30) and indicates a simpler genetic determination of mating in Streptomyces (22,25).

"Fertility"

A "cross" in Streptomyces does not lend itself to kinetic analysis. It is a mixed culture of 2 parental strains growing on agar for a period of several days, during which a new generation of spores is produced. Among these spores, new genotypes are detected by inoculating samples on selective plating media. Most of the

Tab. 1. Small self-transmissible Streptomyces plasmids.

Plasmid	Size (kb)	Upper limit of transfer and Ltz region (kb)	Reference
pIJ101	8.9	3.1	22
SLP1.2	14.5	5	Kieser (unpublished)
SCP2*	31	10	19
pSF765	7.2	7.2	47
pNM100	9.3	9.3	23
pJV1	10.8	10.8	48
pVE1	11	11	49

Tab. 2. Basic replicons of Streptomyces plasmids.

Plasmid	Copy no.	Size (kb)	Upper limit of replicon (kb)[a]	References
pIJ101	40-300	8.9	2.05	22
SLP1.2	4-5	14.5	2.7 + 1.4[b]	Kieser (unpublished)
SCP2*	1-2	31	5.5	19, 50
pFJ103	(High)	20	2.9	51
pVE1	(High)	11	3.3	49
pNM100	c.600	9.3	4.0	23
pSK1	(High)	10.3	3.8	52
pSK2	>20	10.3	2.9	52

[a] In most cases the results are quite peliminary; therefore these values may be considerable over-estimates

[b] Two discontinuous regions of essential DNA

self-transmissible Streptomyces plasmids that have been examined promote the recombination of host chromosomal markers in such crosses. Several plasmids have been tested in a common background: genetically marked strains of S. lividans 66 carrying no other known plasmids (31). In crosses of a plasmid-carrying with a plasmid-free strain, the frequency of recombinants (selecting for one marker from each parent) as a fraction of the total spores varied reproducibly with the plasmid from > 10^{-3} for pIJ101 to < 10^{-5} for SCP1 and pJV1, with other plasmids having an intermediate fertility of around 10^{-4} (Tab. 3). On the other hand, SLP3 and SLP4 do not appear to act as fertility factors. The variation in fertility did not appear to reflect differences in the frequency with which the plasmids were acquired by the initially plasmid-free strain, since all the plasmids were transferred to at least 50% and usually close to 100% of the other parent. This frequency is not, however, a measure of the primary transfer of the plasmids but also depends on secondary transfer within the recipient culture (see below). For SLP2 and pJV1 in Tab. 3 (as well as SCP2 in S. coelicolor A3(2) and SRP1 in S. rimosus; Ref. 32, 33) fertility was significantly reduced when the same plasmid was present in both parents. This effect may be related to "entry disadvantage" (34) or "exclusion" (19), terms describing the failure of plasmid markers to reassort freely with host chromosomal genotypes in crosses between strains carrying marked plasmids. The

Tab. 3. Chromosomal recombination frequencies in crosses between S. lividans strains caused by various plasmids.

Plasmid	Recombinant fraction: Plasmid$^+$ X Plasmid$^-$	Recombinant fraction: Plasmid$^+$ X Plasmid$^+$
SLP3	$< 2 \times 10^{-9}$	-
SLP4	$< 3 \times 10^{-9}$	-
SCP1	5.1×10^{-6}	3.2×10^{-6}
SCP2*	1.2×10^{-4}	1.0×10^{-4}
SLP2	2.5×10^{-4}	1.8×10^{-6}
pIJ6 (from SLP1.2)	1.4×10^{-4}	1.9×10^{-4}
pIJ303 (from pIJ101)	4.3×10^{-3}	4.3×10^{-3}
pIJ110	1.0×10^{-4}	1.2×10^{-4}
pIJ408	3.0×10^{-4}	2.2×10^{-4}
pIJ928 (from pIJ107)	5.3×10^{-5}	4.3×10^{-5}
pWOR191 (from pJV1)	5.1×10^{-6}	$c.1 \times 10^{-8}$

Data from ref. 31 except pIJ928 (19) and pWOR191 (48).

explanation of such "exclusion" is not known, but it might include the effects of any one, or a combination, of several factors, such as processes analogous to the surface exclusion and incompatibility shown by plasmids of gram-negative bacteria, as well as phenomena that may be peculiar to Streptomyces (7).

Patterns of Marker Inheritance in Crosses Involving SCP1

In crosses between SCP1$^+$ strains of S. coelicolor (presumed to carry autonomous SCP1) and SCP1$^-$ strains, there was a tendency for recombinants to inherit markers preferentially from the SCP1$^+$ parent (35). This result is compatible with the presence, within an SCP1$^+$ culture, of high-frequency donors which contribute markers preferentially to the progeny (rather as the fertility of F$^+$ cultures of E. coli depends partially on a minority of Hfr bacteria in the F$^+$ population; Ref. 36). Various types of SCP1-donor strains have been isolated from SCP1$^+$ cultures; these are listed in Tab. 4. For most of the donor types, the donor character was stable during subculture, but in a few cases it was highly unstable. In crosses of any of the donor types with an SCP1$^-$ strain, essentially 100% of the previously SCP1$^-$ genotype (taking account of the instability of the donor character where appropriate) were found to acquire donor properties apparently identical with those of the donor parent, but the patterns of inheritance of donor chromosomal markers varies with the strain (Fig. 1).

Tab. 4. Donor strains of S. coelicolor A3(2) involving SCP1.

Donor type	Origin	Characteristics	Reference
NF	SCP1$^+$ X SCP1$^+$ mating	Bidirectional donor from 9 o'clock position; integration of SCP1 into chromosome inactivated dagA	37, 53
NF-like donor (A634)	Unstable donor A620 crossed with SCP1$^-$	Bidirectional donor from near 9 o'clock position (but dag$^+$)	38
Bidirectional donor	SCP1'-cysB	Bidirectional donor from near cysB	39
Unstable "pabA" donors (e.g. A607, A620); Unstable "uraA" donor (A610); Stable "pabA" donor (A608); Stable "cysD" donor (2106)	SCP1$^+$ strain	Unidirectional donors from three different sites	38
SCP1'-cysB; SCP1'-argA, uraB	SCP1$^+$	Primes carrying chromosomal genes	39

Markers were inherited from 2 of the stable donors (A608 and 2106) with a gradient of frequencies falling with increasing distance, in one direction only, from a fixed point on the linkage map. Such "unidirectional" donation of markers is reminiscent of the behavior of Hfr donors in E. coli which result from covalent integration of a plasmid into the chromosome; the main difference is the inheritance of the donor character by all recombinants, including those acquiring only markers close to the "integration" point. Three other stable donors--the original NF type and 2 donors derived secondarily from apparently autonomous plasmid variants [the NF-like donor A634 (Ref. 38) and the bidirectional donor derived from SCP1'-cysB (Ref. 39)]--showed a "bidirectional" gradient of marker donation. All these stable donors are likely to have originated by integration of SCP1 into the chromosome, and this idea is supported by 2 specific observations. 1) NF strains lack the activity of a chromosomal gene, dagA, which determines a diffusible agarase and which maps at the 9 o'clock position on the chromosome (37), the same point as the center of the bidirectional gradient of marker donation; it is postulated that dagA was inactivated by integration of SCP1. 2) The bidirectional donor derived from an SCP1-prime strain in which the plasmid carries the cysB gene donated

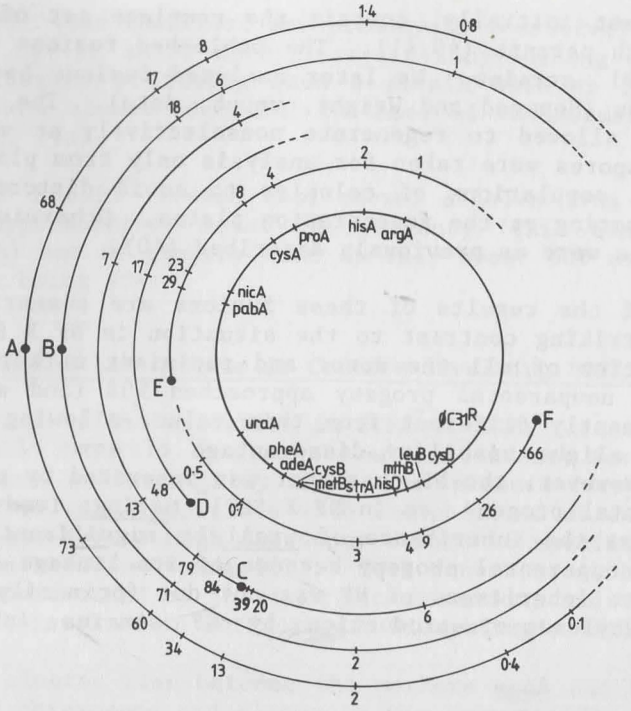

Fig. 1. Percentage frequencies of inheritance of donor markers by recombination in matings of various types of S. coelicolor SCP1-donors with SCP1$^-$ strains. A: NF (from Ref. 53); B: NF-like donor A634 (from Ref. 39); C: bidirectional donor derived from SCP1'-cysB (from Ref. 38); D: unstable "uraA" donor A610 (from Ref. 39); E: unstable "pabA" donor A607 and stable "pabA" donor A608 (from Ref. 39); F: stable "cysD" donor 2106 (from Ref. 13). The ● symbol indicates the presumed position of SCP1, inherited by approximately 100% of the recombinant progeny.

chromosomal markers with a gradient centered on this locus (39); integration via homologous recombination is the most likely explanation for the origin of this donor, and the slight instability of the donor property (∿ 0.1%) is compatible with re-excision of the plasmid by recombination between the tandemly repeated copies of the chromosomal segment which would be present.

Protoplast Fusions Between NF and SCP1$^-$ Strains

In protoplast fusions between pairs of S. coelicolor strains, recombinants originate at a high frequency. It can be deduced that they arise by repeated rounds of recombination within fusion bodies

that, at least initially, contain the complete set of chromosomal genes of both parents (40,41). The published fusions were between pairs of SCP1⁻ strains. We later analyzed fusions between NF and SCP1⁻ strains (Hopwood and Wright, unpub. data). The fused protoplasts were allowed to regenerate nonselectively at varying dilutions, and spores were taken for analysis only from plates carrying subconfluent populations of colonies to avoid distortions of the results by mating on the regeneration plates. Otherwise, the technical details were as previously described (40).

Some of the results of these fusions are summarized in Fig. 2(a). In striking contrast to the situation in NF X SCP1⁻ matings the frequencies of all the donor and recipient markers (except at uraA) among nonparental progeny approached 50% (and were probably not significantly different from this value, allowing for the effects of a slight viability disadvantage of some of the alleles; Ref. 40). However, the NF character was inherited by up to 100% of the nonparental progeny, as in NF X SCP1⁻ matings (and this presumably explains the inheritance of uraA1 by significantly more than 50% of the nonparental progeny because of its linkage to NF). That this obligate inheritance of NF was not due (primarily) to the effect of methylenomycin-production by NF strains inhibiting the

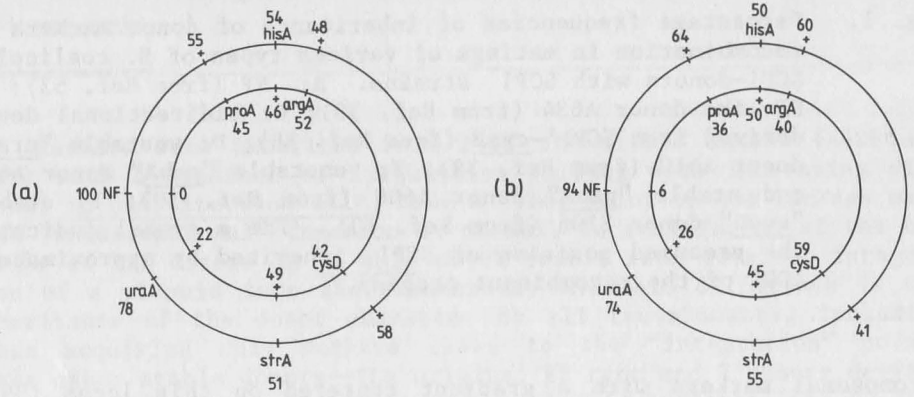

Fig. 2. Percentage inheritance of markers and of the NF character by recombinants in protoplast fusions of NF and SCP1⁻ strains of S. coelicolor. (a) NF strain A317 hisA1 uraA1 strA1; SCPT strain A700 proA1 argA1 cysD18 (b) NF strain 2439 hisA1 uraA1 strA1 (methylenomycin nonproducing mutant R11: Ref.42); SCP1⁻ strain A700 proA1 argA1 cysD18. In (a), there were 146 recombinants, representing 27.4% of the total progeny (which also included 316 of the NF parental genotype and 51 of the SCP1⁻ parental genotype); in (b) there were 102 recombinants, representing 27.3% of the total progeny (225 NF parentals and 46 SCP1⁻ parentals).

growth of SCP1⁻ (and therefore methylenomycin-sensitive) strains was shown by the use of an NF methylenomycin nonproducing mutant (mmy) (42). The results of fusing such a strain with an SCP1⁻ strain [Fig. 2(b)] were almost identical to those of the normal NF X SCP1⁻ fusion in Fig. 2(a).

In an NF X SCP1⁺ fusion (not shown) alleles from each parent were again approximately equal in frequency (this time including those at uraA) but NF progeny made up only about 50% of the total, the remainder being SCP1⁺.

Patterns of Marker Inheritance in Crosses Involving SCP2*-Primes

In contrast to the situation in SCP1⁺ X SCP1⁻ crosses, SCP2⁺ X SCP2⁻ (or SCP2* X SCP2⁻) crosses showed no tendency for progeny to inherit markers preferentially from the plasmid-carrying parent (32). This result is compatible with the observed absence (or perhaps extreme rarity) of high frequency donors of specific groups of chromosomal markers involving SCP2. Such donors have, however, recently been constructed by in vitro recombination, and include some containing DNA involved in actinorhodin biosynthesis (20).

The act cluster lies between the markers guaA and hisD on the S. coelicolor chromosome and closer to the latter (43). The entire set of act genes, and subsets of them, were cloned on to a derivative of SCP2* called pIJ922 (Fig. 3) which appears to be identical to SCP2* in its transfer and fertility properties (20). These clones, and subclones derived from them, provide a series of artificial SCP2*-primes with chromosomal segments of varying length inserted in either direction into the plasmid, all at the same site. Each clone, as well as pIJ922 itself, was introduced into an SCP1⁻ SCP2⁻ strain of S. coelicolor and the resulting strains were each crossed with an SCP1⁻ SCP2⁻ strain carrying a complementary set of chromosomal markers. The markers were chosen in such a way that the donation by the plasmid-carrying strain of alleles at loci (hisD, guaA, strA, and argA) situated at varying distances from the act region could be studied, with counter-selection at a distant locus, proA. The frequencies of donation of the markers are plotted in Fig. 4 as a function of the distance of the markers from act.

The main features of the results are as follows: 1) Whereas pIJ922 promoted the transfer of all 4 markers at a frequency of about 10^{-3}, characteristic of SCP2* X SCP2⁻ crosses in S. coelicolor (32), the primes carrying inserts of chromosomal DNA at least 9.5 kb long caused increased donation of markers, at a frequency that fell with increasing distance of the marker from act. 2) The orientation of the inserts into the plasmid did not affect the pattern of marker donation (thus pIJ2300 and pIJ2302 have one orientation and pIJ2301 and pIJ2303 have the other); this is particularly telling for hisD⁺ and guaA⁺ which lie close to act and on opposite sides of

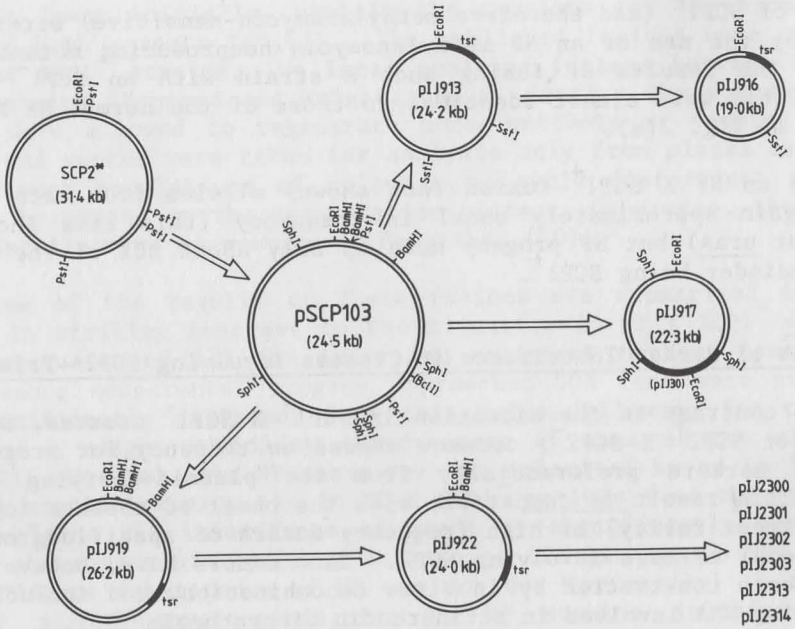

Fig. 3. Derivation from SCP2*, by in vitro deletions and additions of DNA, of the plasmids referred to in the text. Note that pSCP103 is drawn on a larger scale and that each plasmid includes only those restriction sites needed to explain the steps in the construction. The PstI C + D fragments of SCP2* were deleted to form pSCP103 (14). The BamHI B fragment of pSCP103 was replaced by the BamHI fragment of pIJ30 that carries tsr (thiostrepton resistance) to form pIJ913. The SstI B fragment of pIJ913 was deleted to form pIJ916. The SphI C + D + E fragments of pSCP103 were replaced by pIJ30 to form pIJ917. The BamHI tsr fragment of pIJ30 was inserted into BclI/ site 12 of pSCP103 (1 of 8 BclI/ sites in that plasmid: Ref. 19) to form pIJ919. The BamHI B + C fragments of pIJ919 were deleted to form pIJ922. Fragments of S. coelicolor chromosomal DNA, carrying various parts of the act gene cluster (20), were inserted into the BamHI site of pIJ922 to form pIJ2300, pIJ2301, pIJ2302, pIJ2303, pIJ2313, and pIJ2314.

it. 3) Recombinants selected to carry hisD$^+$ (or guaA$^+$), on one side of act, also inherited guaA$^+$ (or hisD$^+$) on the other side of act at a high frequency (25-75%) irrespective of the orientation of the chromosomal insert in the plasmid. 4) In all the crosses, the plasmids were transferred to 40-100% of the recipient (initially SCP2$^-$) strain.

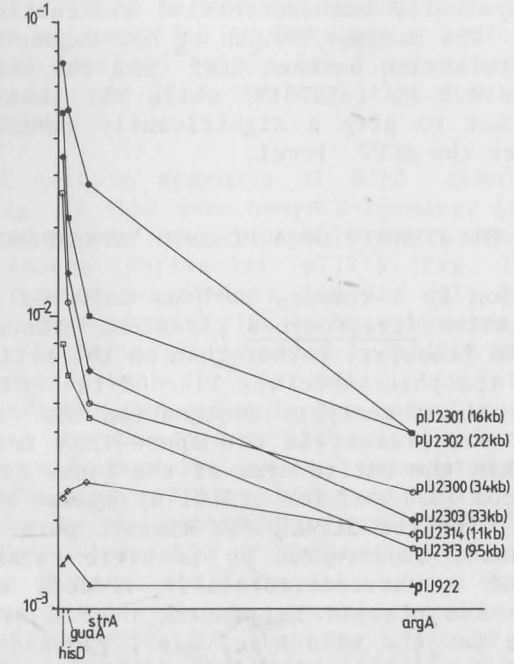

Fig. 4. Frequencies of inheritance of markers in matings of donor S. coelicolor strains carrying clones with chromosomal segments of varying length inserted into pIJ922 (and pIJ922 itself) crossed with SCP1$^-$ strain B341. Ordinate: frequency of each donor marker among recombinants, expressed relative to the SCP1$^-$ parental genotype. Abcissa: relative distance of each of the four markers from the act cluster. Numbers in parentheses are the sizes of the chromosomal inserts in each clone in kilobase pairs (the inserts in pIJ2300 and pIJ2302 had one orientation in the vector, while those in pIJ2301 and pIJ2303 had the opposite orientation). See text for further details.

These results indicate that homology between the plasmid and the chromosome can lead to an enhanced frequency of transfer of chromosomal markers, specifically of those close to the region of homology. This probably arises from an integration of the plasmid via homologous recombination to give a high frequency donor state. If so, the orientation of the integrated plasmid does not impose a polarity on the pattern of marker donation; the same, bidirectional, gradient results irrespective of orientation. (This is unlikely to arise from a combination of 2 unidirectional gradients, since markers on either side of the region of homology are often inherited together.) These are the same features as those observed in crosses

involving the physically uncharacterized bidirectional SCP1 donors mentioned above. The minimum length of homologous DNA required for a significant interaction between SCP2* and the chromosome is certainly less than 9.5 kb (pIJ2313) while the insert of 1.1 kb in pIJ2314 appears not to give a significantly enhanced frequency of recombination over the SCP2* level.

POCK FORMATION: THE CONCEPT OF A PLASMID "SPREADING" FUNCTION

Pock formation is a common, perhaps universal, phenotype conferred by conjugative Streptomyces plasmids. Since pock formation depends on plasmid transfer, rather than on the action of diffusible inhibitors (18), the phenomenon was likened to lethal zygosis in E. coli (44); hence the phenotypic designation Ltz$^+$ (25). Copies of the plasmid that was present in the spore that initiated the pock can be found within the entire area of the pock, as shown for SCP2* by Bibb and Hopwood (32) and for pIJ101 by Kieser et al. (22). For pIJ101 (22), and also for SLP1.2 (T. Kieser, pers. comm.), a class of plasmid variants, constructed by in vitro recombination, gives rise to pocks of a characteristically reduced size, designated Ltz$^{+/-}$, in which the plasmid is present over a much smaller area than is the case for the wild-type, Ltz$^+$, plasmids (Fig. 5). The discovery of such Ltz$^{+/-}$ plasmids for pIJ101 gave rise to the concept of an active plasmid-determined spreading function, distinct from the ability to promote mating since the ability of Ltz$^{+/-}$ plasmids to be transferred in a mass mating appeared to be unimpaired. In vitro deletion or insertion of DNA into a region of the plasmid distinct from that involved in the Ltz$^{+/-}$ phenotype abolished pock formation completely and also rendered the plasmids incapable of transfer in matings.

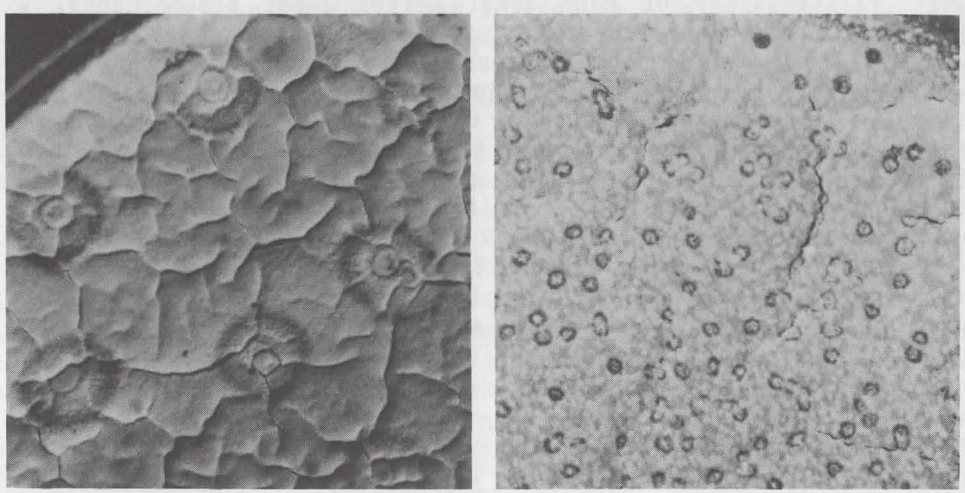

Fig. 5. Pocks produced by derivatives of pIJ101 in lawns of plasmid-free S. lividans. Left: normal sized pocks (Ltz$^+$) produced by pIJ303; right: small pocks (Ltz$^{+/-}$) produced by pIJ224. See Ref. 22 for details.

Deletion of certain segments of SCP2* resulted in plasmids (e.g., pIJ917, Fig. 3) that were nonpock-forming, transfer-deficient (10^{-5} compared with 1), and incapable of promoting chromosomal recombination. Another derivative, pIJ916 (Fig. 3), was also nonpock-forming and transfer-deficient, but had only a slightly reduced fertility (10^{-5} compared with 10^{-4}) in otherwise plasmid-free S. lividans. Thus, the fertility promoted by pIJ916 was close to unity per transfer, compared with 10^{-4} for SCP2*. Lydiate (19) suggested that pIJ916 was totally defective in the normal spreading function. As a consequence, one observed a high value of recombination per transfer because all transfer events were primary intermycelial events, rather than many of them being secondary events caused by plasmid spread within the genetically uniform SCP2$^-$ recipient culture and, therefore, incapable of generating recombinants. It appears, then, that primary transfer events have a very high probability of being associated with chromosomal recombination. [The very high frequency of recombination that was observed in the center of SCP2* pocks studied by Bibb and Hopwood (32) may also be a reflection of this. However, the strains in this study also carried SCP1 and the pocks may not be typical; further work will be needed to test the generality of this result.]

DISCUSSION: TRANSFER, SPREADING, AND FERTILITY OF STREPTOMYCES PLASMIDS

One of the most striking characteristics of conjugative Streptomyces plasmids is the small size of the segment of plasmid DNA required for the expression of conjugal properties; in the most studied example, pIJ101, this is not more than about 2 kb. Of course, the chromosome of the host (or perhaps other unrecognized plasmids) may provide functions in trans that are involved in conjugation; in that case, such functions are likely to be generalized in Streptomyces since pIJ101 has been found to be conjugative in several host species including strains of S. lividans which do not reveal evidence of the presence of any other plasmid. Plasmid transfer is, however, not entirely passive, since transfer-defective plasmid variants occur. Wild-type pIJ101 and several unrelated plasmids promote the transfer of transfer-defective pIJ101 derivatives at the same rather low level as they promote the transfer of chromosomal markers (22). This argues for a cis-acting function required for transfer of plasmid DNA and defective or absent in the nontransferable derivatives, all except one of which were known to have deletions of DNA including the transfer region (and the exception may

well have had a small unrecognized deletion or insertion into the cis-acting site). The requirement of one or more (but not many) trans-acting plasmid-coded gene products for transfer is not excluded, but probably host gene products predominate in establishing the mating unions between hyphae; a purely host-determined hyphal fusion may perhaps provide the channel through which DNA is transferred.

In the cases where we have knowledge of the plasmid status of the host strains -- primarily S. coelicolor and S. lividans -- the transfer of chromosomal DNA (as measured by recombination of markers) does not occur, or occurs only at an extremely low level, in the absence of plasmids. The evidence is clearest for S. lividans in which we were unable to detect definitive recombinants in SLP2$^-$ SLP3$^-$ strains (22,31); a low, variable level of nonparental colonies arose, but those tested had the genotypes that would have been expected to result from occasional mutation of a marker. In S. coelicolor, SCP1$^-$ SCP2$^-$ strains do yield a low level of true recombinants ($\sim 10^{-8}$; Ref. 32) but, since these strains harbor the integrated form of SLP1, which certainly promotes fertility in its autonomous form in S. lividans (31,32), such recombinants could be plasmid-dependent. A wide variety of plasmids stimulate fertility, and to levels characteristic of each plasmid, the highest values reaching several percent (22). Moreover, some pairs of strains are less fertile when the plasmid is present in both strains than when it is present in only one (Tab. 3). These observations indicate that plasmids are involved in the transfer and/or recombination of chromosomal DNA. [The high frequency of recombination in protoplast fusions between plasmid-free strains (40) might argue against a role for plasmids in recombination itself, but it may be that protoplast formation or fusion disrupts a normal compartmentation of chromosomes which limits recombination.] However, a minority of plasmids, currently represented by SLP3 and SLP4 (31), does not promote the transfer of chromosomal DNA. Thus, chromosome transfer is not an inevitable consequence of plasmid transfer. Specific plasmid functions leading to fertility should therefore exist, but have not yet been demonstrated. In the case of SCP1, chromosome transfer could result from covalent association between plasmid and chromosomal DNA in various donor states, which presumably arise via recombination within special DNA sequences. This seems unlikely for SCP2. For other plasmids, no attempts to identify such donor states have been reported.

The analysis of genotypes within pocks, and the existence of the Ltz$^{+/-}$ class of plasmid variants (which are still fully transferable and promote normal high levels of fertility in mass matings), points to the existence of a "spreading" ability possessed by pock-forming conjugative plasmids. The mechanism of such spreading is unknown, but it has been suggested (22) that it might occur via intramycelial migration of plasmid DNA copies after primary intermycelial transfer by mating. We have postulated that, after primary

transfer between mating individuals, the plasmid migrates away from the pock center, within mycelium exclusively containing chromosomes of recipient genotype. Thus, even if chromosomal DNA were mobilized during such migration, few further recombinants would be generated because the situation would be equivalent to constant "backcrossing" (45). A variant such as pIJ916, showing an abnormally high recombination frequency per transfer, is believed to be unable to spread; therefore, all transfers would be primary events and no such backcrossing would occur. While conduction of chromosomal DNA may be possible via some process not requiring covalent association between plasmid and chromosomal DNA, the high frequency of donor markers that results specifically from the presence of DNA homology between chromosome and plasmid in the SCP2*-primes suggests that this can be an important factor in the process.

The gradients of marker inheritance by recombinants in crosses involving some of the donor states of SCP1, and the artificial SCP2*-primes, might result from backcrossing. Thus, even if complete chromosomes were transferred via the mating reaction, continued conduction of chromosomal DNA during plasmid migration, and recombination with recipient chromosomes en route, would progressively dilute donor markers; and the more so, the looser their linkage with the point of association of donor DNA with the plasmid responsible for its conduction. The steepness of the gradient for a given set of markers would depend on the extent of migration compared with the frequency of primary plasmid transfer. The absence of a gradient in protoplast fusions between NF and SCP1 strains (except for markers close to the SCP1 integration site) would follow from the bringing together of statistically equal numbers of entire genomes of each parent into each fusion body; therefore, there would be no "backcrossing."

The donor state is inherited by almost all progeny of crosses of highly fertile strains carrying SCP1 variants with plasmid-free strains -- and also of fusions of NF with SCP1$^-$ strains -- whereas, crosses and fusions with SCP1$^+$ strains lead to a segregation of donor and SCP1$^+$ types. We have no unique explanation for this finding. However, it may result in part from the efficient spreading of plasmid copies, including those integrated into the chromosome, through the substrate mycelium, combined, perhaps, with an efficient mechanism that tends to ensure the inclusion of a plasmid copy in every spore. Possibly the development of mycelium and/or spores is inhibited in regions in which too few copies of SCP1 exist to allow their inheritance by every cellular compartment or spore, a phenomenon that would be analogous to the coupling of host-cell division to plasmid proliferation exhibited by F (46). When both variant and normal forms of SCP1 are present together, a segregation of plasmid copies would result in the observed inheritance of either plasmid form.

There remains the problem of explaining the contrast between the bidirectional gradients of marker inheritance seen in crosses involving the SCP2* -primes and some of the SCP1 donors, and the unidirectional gradients shown by other SCP1 donors. The bidirectional donation of markers could be explained by a unidirectional single-stranded DNA transfer mechanism similar to that revealed for Hfr forms in E. coli, assuming selection for the integrated plasmid and transfer of a DNA strand longer than the entire chromosome. This explanation can be extended to the unidirectional donor types if a hot spot for recombination is postulated to exist close to one side of the integrated plasmid. An attractive, alternative model for the bidirectional donation of markers involves the possible transfer of Streptomyces plasmids as double-stranded circular molecules. Transfer of a plasmid integrated into the chromosome would then result in the passive conduction of the whole chromosome. Repeated rounds of recombination and plasmid spread would result in recombinants where donor alleles predominate at loci close to the site of plasmid integration, and recipient alleles would predominate at loci that are distant from the site of plasmid integration. Again, a hot spot for recombination close to one end of the integrated plasmid would explain the unidirectional donation of markers. At present, it is impossible to choose between these alternatives but it is to be hoped that future work, largely made possible by the improved techniques now available for analysis and manipulation of Streptomyces DNA, will shortly throw more light on the biology of the interesting conjugative plasmids of this group of bacteria.

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THE STRUCTURE AND SOURCE OF PLASMID DNA DETERMINE THE CLONING PROPERTIES OF VECTORS FOR BACILLUS SUBTILIS

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INTRODUCTION

The elaboration of a system for cloning in bacilli began with a search for plasmids capable of replicating in Bacillus subtilis cells. Various strains of bacilli often carry such plasmids, but most of them are either cryptic or their markers are not selective enough (for review, see Ref. 1,2). Although these plasmids are usable as vectors (2), major progress in gene engineering with bacilli is associated with the use of vectors originating from a phylogenetically different group of organisms. It was found that staphylococcal plasmids can transform B. subtilis cells and render them drug-resistant (3). The plasmids are maintained in a large number of copies in B. subtilis and carry some unique restriction sites suitable for cloning (4,5,6). Several genes were successfully cloned on staphylococcal plasmids as vectors, although as experimentation was extended, it was found that the efficiency of cloning in B. subtilis is much lower than in the traditional Escherichia coli system (4,7,8). Part of the reason for that has become clear after it was demonstrated that B. subtilis, unlike E. coli, cannot be transformed with plasmid monomers which constitute the bulk of plasmid populations isolated from cells or produced in routine in vitro ligation of DNA fragments. Accordingly, staphylococcal plasmids were found to transform B. subtilis only as oligomers (9,10), although both oligomers and monomers penetrate into the cell equally well. The plasmid enters the B. subtilis cell as a linear, single-stranded molecule (11), and the possibility of transformation is largely dependent on whether recycling of the strand can occur. The latter, in turn, is believed to depend on the presence of genome redundancy in the plasmid. Thus, the transforming efficiency of plasmid

oligomers is associated with the existence of repeated sequences in them needed for the closure of the penetrated strand (11,12). This implies that transformation of B. subtilis with recombinant plasmids obtained by in vitro ligation could be accomplished through the use of special methods yielding oligomers of these plasmids. Success along these lines came with the creation of shuttle vectors capable of propagation both in B. subtilis and in E. coli (4,13,14). Recombinant plasmids based on these vectors are first accumulated in E. coli, and the DNA preparation obtained already containing plasmid oligomers, is used to introduce the plasmids into B. subtilis. The employment of E. coli as an intermediate host has greatly extended the potential of gene engineering in bacilli with regard to cloning individual DNA fragments (15,16,17,18) and creating a library of genes (19,20).

Another line of research was concerned with devising methods for transformation of bacilli with monomers of recombinant plasmids. One such method is "marker rescue", which involves recombination of a monomeric plasmid with a homologous region of a resident plasmid (21,22). It was found that the rescue could also occur on a homologous region of the host chromosome (23,24). Plasmid monomers lacking a region homologous to the recipient DNA can also be transformationally active if they carry direct repeats (25). Yet another approach to introducing plasmid monomers into bacilli consists in using PEG-dependent transformation of protoplasts (26). The development of the various techniques for cloning in B. subtilis has made this system more competitive with regard to the E. coli system, and it is now widely used for cloning homologous and heterologous genes (for review, see Ref. 27,28).

Some problems remain, however, and one of them is cloning of large (over 3 Mdal) fragments on staphylococcal plasmids or their derivatives. Cloned fragments in gene libraries obtained from B. subtilis are usually smaller than 3 Mdal (8,17,29), and when a mixture of fragments is subject to cloning, the heavier ones become underrepresented (30). We also experienced a problem when we attempted to propagate in B. subtilis a riboflavin operon within a 9.5-kb (6.3-Mdal) EcoRI fragment. A search for ways to overcome this problem has revealed some peculiarities of B. subtilis transformation with plasmid DNA, and vectors have eventually been constructed that offer effective cloning of relatively large DNA fragments.

Section 1. Characteristics of the Rib Fragment

Genes of riboflavin biosynthesis in B. subtilis are organized into an operon located between lys and ser markers (210°) on the chromosome map (31). The operon is repressed by the product of the regulator gene situated outside of this linkage group (32). The

operon was found to contain a ribO operator locus whose mutations may cause derepression of the operon and overproduction of riboflavin (32,33). As a source of the rib operon we used 2 B. subtilis strains: SHgw with the wild-type operon($ribO^+$) and CHP335 with a derepressed rib operon (rib0335 = $ribO^c$) (Tab. 1).

Both chromosomal DNAs were treated with EcoRI, and the fragments obtained were cloned in E. coli $r^-_k m^+_k$ on the RSF2124 and pBR322 vectors. The resulting plasmids were designated pPR2 (RSF2124-Rib(O^c)), pLP95 (pBR322-Rib(O^c)) and pLP102 (pBR322-Rib(O^+)) (35,36). The plasmid pLP102 carries the wild-type operon and pPR2 and pLP95 carry the derepressed operon (Tab. 2). The Rib fragments in all the plasmids have the same size, 9.5 kb, and produce a similar restriction pattern (36). The rib genes of B. subtilis within all 3 plasmids are expressed in E. coli and complement the appropriate auxotrophic mutations in this host (17,39).

Section 2. Cloning of Rib Fragments with the Aid of the pUB110 Replicon

The plasmid pUB110 (4.5 kb), originating from Staphylococcus aureus (5), proved to be a good vector for B. subtilis in a number of cases (7,12,40). It codes for kanamycin resistance and has unique EcoRI and BamI sites suitable for cloning.

We made several attempts to propagate the Rib fragment on pUB110 in shotgun experiments, as well as to reclone it from plasmid pPR2. The chromosomal DNA of B. subtilis or the pPR2 DNA were treated with EcoRI and ligated with pUB110 linearized with the same enzyme. The ligation product was used for transformation of B. subtilis ribD107recE4 cells (Tab. 1). The recE4 mutation served to

Tab. 1. Bacteria.

Strain	Genotype	Source
Bacillus subtilis		
SHgw ($rib0^+$)	prototroph	T.P.Chernik
CHP335 ($rib0^c$)	rib0335	- " -
107 (rec^+)	ribD107	- " -
PG260 (recE4)	ribD107 recE4	(18)
Escherichia coli		
BSV82 (rib82)	thi rib82 strA hsdR $hsdM^+$	(34)
BSV821 (rib82 recA)	the same as BSV82 and recA srl::Tn10	(38)

Tab. 2. Plasmids.

Plasmid	Phenotype	Size (kb)	Source
pBR322	Ap Tc	4.3	G.Boyer
pPR2	Ap Rib(O^c)	20.3	(35)
pLP95	Ap Tc Rib (O^c)	13.8	(36)
pLP102	Ap Tc Rib(O^+)	13.8	- " -
pUB110	Km	4.5	D.Dubnau
pJJ1	Ap Km	8.7	(14)
pMX1	Ap Km Rib(O^+)	22.2	(37)
pMX9	Ap Km Rib(O^+)	18.3	- " -
pMX10	Km	4.1	- " -
pMX30	Em	18.3	- " -
pMX33	Em Rib(O^+)	27.8	(38)
pMX49	Em Rib(O^c)	27.8	This study
pMX39	Ap Em	22.8	(38)
pMX121	Ap Em	6.1	This study
pPIE10	Ap Em	9.5	A.S.Boitsov
pLA12	Ap Em	12.8	This study
pLA32	Ap Em	8.8	- " -
pLA110	Ap Em	9.1	- " -
pLA1718	Ap Em	9.4	- " -
pLA44	Ap Em Rib(O^c)	18.6	- " -

prevent recombination between homologous regions in the plasmid and chromosome. The transformants selected by the $Km^r Rib^+$ phenotype were used to isolate plasmid DNA which was tested for presence of the recombinant plasmids. No such plasmids were found, unfortunately, but the transformants, instead, contained small plasmids, similar in size to pUB110 and carrying only the Km^r marker. The Rib^+ phenotype of the transformants at first seemed mysterious to us. However, the evidence to be presented below allows the conclusion that this phenotype is due to a pUB110-Rib plasmid present in very few copies in the selected population and thus undetectable by the methods employed in the study.

In the next series of experiments, we attempted to clone the Rib fragment using E. coli as an intermediate host. We began with construction of a few plasmids each of which contained pUB110 DNA and the Rib fragment within the RSF2124 or pBR322 replicon. Neither

of these plasmids was able to transform B. subtilis (17,37,38). Then we decided to expand the search for recombinant plasmid configurations in E. coli. The scheme of the experiment is depicted in Fig. 1.

The shuttle vector pJJ1 (pBR322-pUB110) (14) was chosen as the parental plasmid. It determines $Ap^r Km^r$ properties in E. coli and the Km^r phenotype in B. subtilis and is cleaved by EcoRI into 2 fragments: fragment A (4.7 kb) which contains mainly the pBR322 DNA, and fragment B (4.1 kb) which contains mainly the pUB110 DNA. The A and B fragments are genetically marked by the Ap^r and Km^r determinants, respectively. The Rib fragment obtained by preparative fractionation of EcoR1 digest of plasmid pLP102 was added to the mixture of A and B fragments (Fig. 1).

The mixture of A, B, and Rib was ligated and used to transform E. coli rib82 cells, with subsequent selection for the Ap^r, Km^r and Rib^+ characters. Plasmids from 11 independent E. coli transformants were checked for the ability to transform B. subtilis ribD107recE4 cells and render them $Km^r Rib^+$. As Tab. 3 shows, 10 plasmid preparations out of the 11 failed to do so, but one (no. 7) did show transformational activity. Electrophoretic and restriction analyses of the plasmid preparations showed that all of them contained an 18.2-kb plasmid, while preparation no. 7 contained an additional 22.2-kb plasmid. Each of the 18.2-kb plasmids was composed of the 3 EcoRI fragments employed (A, B and Rib), their mutual orientation

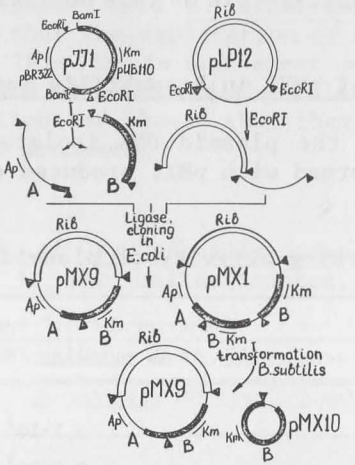

Fig. 1. Construction of plasmid pMX1 containing direct DNA repeats. (————) - pBR322, (▬▬) - pUB110, (▭▭) - Rib, (▼) - EcoRI sites; A, B - EcoRI fragments of pJJ1 plasmid.

Tab. 3. Transforming activity of plasmids containing EcoRI fragments A and B of plasmid pJJ1 and the Rib fragment.

Recipient	Selective markers	Number of transformants per μg DNA	
		Preparation no.1[a]	Preparation no.7
E. coli BSV82 (rib82)	Ap^rRib^+	$7.2 \cdot 10^4$	$9.7 \cdot 10^4$
B. subtilis PG260 (ribD107 recE4)	Km^rRib^+	0	$2.6 \cdot 10^3$

Note:[a] Plasmid preparations no.2 through no.6 and no.8 through no.11 transform E. coli and B. subtilis similarly to preparation no.1

varying as could be expected, from the random ligation of the fragments in vitro. In order to analyze the heterogeneous preparation No. 7, we separated it into the heavier and lighter plasmids by means of transformation and cloning in E. coli rib82recA. The individual plasmids thus obtained were designated pMX1 and pMX9, respectively. Preparations containing pMX1, pMX9, or a mixture thereof, as well as the initial preparation no. 7, were all used to transform B. subtilis ribD107recE4 cells. It was found, as a result, that the transforming activity of preparation no.7 is due entirely to plasmid pMX1 (Tab. 4). Schemes of pMX1 and pMX9 are shown in Fig. 1. The only difference between them is a duplication of segment B in pMX1, which suggests an essential role of this duplication for the transformation of B. subtilis.

Section 3. The Fate of pMX1 in B. subtilis Transformants

To our surprise, the plasmid DNA isolated from Km^rRib^+ clones of B. subtilis transformed with pMX1 produced only 1 electrophoretic

Tab. 4. Transforming activity of plasmids pMX1 and pMX9.

Plasmid	Number of Km^rRib^+ transformants per μg DNA in B. subtilis PG260 (ribD107 recE4)
pMX1	$3.1 \cdot 10^3$
pMX1 + pMX9	$2.1 \cdot 10^3$
Preparation no.7	$2.2 \cdot 10^3$
pMX9	0
pMX1 (monomer)	$2.2 \cdot 10^3$

band corresponding to a 4.1-kb plasmid. Restriction analysis suggested that this plasmid, designated pMX10, is a circular form of the B segment of pMX1 (Fig. 1). It could be assumed that pMX10, which carried the Km^r determinant, appeared in the cell as a result of intramolecular recombination of pMX1. The other product of the recombination then must be identical to pMX9 which carried the other selective marker, Rib^+. The absence of the appropriate heavy band on the electrophoregram could be due to a low copy number of pMX9 in bacilli. In order to demonstrate its presence in the B. subtilis transformants, we recloned the plasmid in E. coli. This was done by transforming E. coli rib82recA cells with the DNA preparation isolated from B. subtilis transformants, followed by selection for Ap^r Km^rRib^+ determinants. All the 3 markers were transferred together, and the plasmid isolated from the E. coli transformants was shown by restriction analysis to be identical with pMX9. The pMX10 DNA, which was the major component of the transforming mixture, could not be maintained in E. coli as it lacked the pBR322 replicator.

Therefore, the plasmid pMX1 which is able to transform bacilli owing to the duplication of the B segment is dissociated in these cells into pMX9 and the circular form of the B segment, pMX10.

Then we isolated a monomer of pMX1 by means of preparative gel electrophoresis. The monomer was as effective for transformation of B. subtilis ribD107 recE4 by Km^rRib^+ markers as the unfractionated preparation (Tab. 5). In both cases the transformants contained pMX10 and pMX9, the latter in a small quantity detectable by recloning in E. coli. Thus, the presence of a repeat in pMX1 ensures transforming activity of the plasmid monomer and allows us to introduce a 9.5-kb Rib fragment into B. subtilis cells. Restriction analysis has indicated that the duplication of the B segment in pMX1 is a direct repeat. This is in agreement with the findings of Michel and coworkers (25) who studied the role of direct repeats in B. subtilis transformation and showed that they conferred transforming activity on plasmid monomers.

Tab. 5. Effectiveness of B. subtilis transformation by pMX30 plasmid DNA in the unfractionated, monomer and linear forms.

Form of plasmid DNA	Number of Em^r transformants per μg DNA	
	B. subtilis 107 (rec^+)	B. subtilis PG260(recE4)
pMX30 unfractionated	$1.2 \cdot 10^3$	$1.5 \cdot 10^4$
pMX30 monomer	$1.2 \cdot 10^3$	$2.9 \cdot 10^3$
pMX30 linearized with EcoRI	0	0

It should be noted that no pMX9 could be visualized in the DNA preparations isolated from B. subtilis transformants. In our opinion, this is due to the fact that the pUB110 vector fails to provide a sufficient copy number of the plasmid in B. subtilis. This assumption may also explain the failure to clone the Rib fragment on this vector. Indeed, the inability of pMX9 and the like plasmids to transform bacilli could well result from their low copy number and thus, the inability to sustain the Km^r phenotype, one of the traits used for transformant selection. The level of kanamycin resistance determined by pUB110 is apparently proportional to the plasmid copy number. As to the Rib^+ trait, it is expressed regardless of rib gene dosage since bacilli cells need but very little riboflavin for their growth. The possibility of selecting pMX1-mediated transformants is due to segregation of a high copy-number plasmid pMX10 which, together with a low copy-number plasmid pMX9, ensures selection of the $Km^r Rib^+$ phenotype.

This segregation of pMX1 induced us to look for vectors able to transform bacilli in the monomeric form and retaining their initial molecular structure at the same time. Studies on the mechanism of B. subtilis transformation with plasmid DNAs (41) led us to assume that plasmids containing long enough inverted repeats would meet this requirement. No such plasmids were found in the above experiments, and we therefore decided to investigate transforming properties of a streptococcal plasmid pSM19035 which was known to carry long inverted repeats.

Section 4. Isolation and Properties of Plasmid pMX30 Containing Inverted Repeats

The plasmid pSM19035 has the size of 27 kb and carries an inducible determinant of erythromycin resistance, Em^r (42). A distinguishing feature of the plasmid are long inverted repeats comprising 80% of the contour length (43). It appears to be identical to another streptococcal plasmid, pSM22095, which is able to transform B. subtilis cells and make them erythromycin-resistant (42,44). We isolated pSM19035 from Streptococcus sanguis (Challis) and tested it in transformation of a B. subtilis recE4 strain. The Em^r transformants obtained had a very low content of pSM19035. We succeeded in isolating a spontaneous deletion derivative of pSM19035, designated pMX30, which had the size of 18.3 kb and was present at 3 copies per genome of bacilli. Based on the restriction map of pSM19035 (42, 45) and on a restriction analysis of pMX30, we constructed a restriction map of pMX30 which is shown in Fig. 2. Electron microscopy indicated that pMX30 retained a portion of the original inverted repeat of pSM19035 which made up about 35% of the pMX30 contour length (Fig. 2).

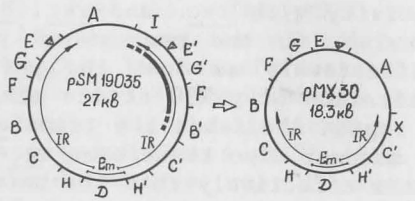

Fig. 2. Structural features of plasmid pMX30. Large letters - HindIII fragments (42,45), (▼)- EcoRI sites. (▬) - deletion, (⟶IR)-inverted repeats.

Tab. 6. Characterization of vector molecules for B. subtilis.

Vector	Insert	Recombinant plasmid	Copy number[a]	Plasmid stability(%)[b]		
				30°	37°	42°
pMX30 18.3 kb	-		3	100	100	100
	Rib(0^+) 9.5 kb	pMX33 27.8 kb	3	100	100	100
	Rib(0^c) 9.5 kb	pMX49 27.8 kb	3	100	100	100
	pBR322 4.3 kb	pMX39 22.8 kb	3	100	100	100
pLA110 9.1 kb	-		35	100	90	<1
	Rib(0^c) 9.5 kb	pLA44 18.6 kb	15	50	30	<1
pLA32 8.8 kb	-		20	80	60	<1
pUB110 4.5 kb	-		40	100	100	100
	Rib(0^+)-pBR322 13.8 kb	pMX9	<1	<1	<1	<1

Notes: a) The copy number was measured as desribed (48). The cells were grown at 37°C.

b) The plasmid stability was determined as described elsewhere (49). The figures correspond to the percentage of clones retaining the plasmid after cultivation for 20 generations under non-selective conditions.

We isolated the pMX30 monomer from the agarose gel and tested its transforming activity with rec$^+$ and rec$^-$ B. subtilis strains. As is evident from Tab. 6, the monomer of pMX30 transforms B. subtilis rec$^+$ as effectively as does the unfractionated plasmid preparation. Linearization of pMX30 at its single EcoRI site outside of the repeat region abolishes the transforming activity completely. The pMX30 monomer also transforms rec$^-$ B. subtilis cells, although 10 times less effectively than the unfractionated preparation (Tab. 6).

We isolated plasmid DNA from rec$^+$ or rec$^-$ B. subtilis cells transformed with either monomers or unfractionated pMX30 preparations and subjected it to electrophoresis and homoduplex analysis. Within the experimental error of these methods, it can be stated that the structure of the plasmids isolated from any of the cell types is the same and identical to that of the initial donor DNA.

Section 5. Vector Properties of pMX30

The ability of the pMX30 monomer to transform B. subtilis cells and to retain its initial structure in the course of transformation makes it a suitable vector for cloning. The pMX30 DNA contains one EcoRI restriction site (Fig. 2). The site is situated outside of the replicative region (45) and may be employed for integration of foreign DNA. Our next experiment, whose schematic representation is shown in Fig. 3, was aimed at finding out whether the pMX30 vector could maintain the Rib fragment in bacilli (see Sec. 1), hoping that the fragment could serve as a probe for testing the vector proficiency of pMX30.

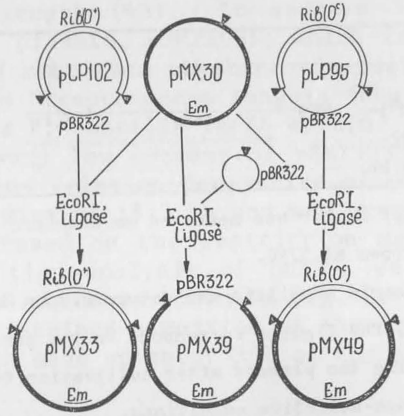

Fig. 3. Hibrid plasmids based on pMX30. (———)-pBR322, (▬)-pMX30, (▭)-Rib, (▼)- EcoRI sites.

The source of the Rib fragment was plasmid pLP102 (pBR322-Rib(0$^+$)) (see Sec. 1, Tab. 2). The pMX30 and pLP102 DNAs were treated with EcoRI, ligated, and the mixture was used to transform B. subtilis ribD107 recE4. The transformants which were selected by the Em^{r}Rib$^+$ markers contained 27-kb pMX30-Rib plasmids, in agreement with the sum of the vector and the Rib fragment masses. One of the recombinant plasmids was designated pMX33 (Fig. 3). A restriction analysis of this plasmid indicated that it contained the pMX30 vector and the entire Rib fragment.

In the next experiment we employed pMX30 to introduce a derepressed rib operon into B. subtilis. The design of the experiment was the same as the above, except the pLP95 plasmid (pBR322-Rib(0^c)) (Sec. 1) was taken as the source of the Rib fragment. The pMX30 and pLP95 plasmids were cleaved with EcoRI, the mixture ligated and used to transform bacilli. The Em^{r}Rib$^+$ transformants displayed 27-kb plasmids, one of which was designated pMX49 (Fig. 3). The restriction pattern produced by pMX49 is indistinguishable from that of pMX33. Phenotypically, however, the 2 plasmids are quite different, since pMX49 determines overproduction of riboflavin in bacilli. Both the plasmids are easily visualized in routine electrophoresis of plasmid DNA, which implies that cloning of the Rib fragment on the pMX30 vector does not lead to a dramatic decrease of the replicon copy number as is the case with the pUB110 vector (Sec. 2,3). In spite of the large insert (9.5 kb), both pMX33 and pMX49 are stably maintained in B. subtilis cells. No loss of the plasmids (< 0.1%) was observed after growing cells harboring them for 20 generations on a medium without erythromycin and supplemented with riboflavin (i.e., without selection for the determinants of the vector and the insert).

Section 6. Construction of the pMX39 Vector on the Basis of Plasmid pMX30

The basic vector properties of pMX30 described above were used in construction of a more convenient shuttle vector. The DNAs of pMX30 and pBR322 were restricted with EcoRI, treated with ligase, and used in the transformation of B. subtilis recE4. Several dozen clones selected by the Em^r phenotype were pooled, and the total DNA isolated from them was used in transformation of E. coli rib82 recA cells, followed by selection for the Ap^r marker of pBR322. The clones obtained carried 22-kb plasmids consisting of pMX30 and pBR322 joined by their EcoRI sites. One of these plasmids was designated pMX39 (Fig. 3). The pBR322 replicon of pMX39 allows the latter to accumulate in an E. coli culture. Experience has shown that preparative isolation of the plasmid from E. coli is more convenient than from B. subtilis, with regard to both yield and purity.

The basic characteristics of the pMX39 vector are as follows: i) unique BamI, PstI, SalGI, and EcoRV sites, and 2 EcoRI sites us-

able for cloning in bacilli; ii) a long inverted repeat important for the transforming properties of the plasmid; iii) stable maintenance of large DNA fragments within plasmids in bacilli.

These properties ensure usefulness of this vector for creating gene banks and in shotgun experiments. In a model experiment we employed pMX39 for cloning fragments of B. amyloliquefaciens DNA in B. subtilis cells. The donor DNA was subjected to limited cleavage with Sau3A restriction endonuclease (average fragment size 15 kb) and then ligated with the DNA of pMX39 linearized at the BamI site. After treatment with ligase, the preparation was used in transformation of B. subtilis amy recE4. The frequency of Em^r transformants was 10^5 per microgram DNA, and Em^r Amy^+ clones appeared among Em^r clones at the frequency of $5 \cdot 10^{-3}$. We isolated plasmid DNA from 10 $Em^r Amy^+$ clones, and the restriction and genetic analyses showed that all of them contained the α-amylase gene of B. amyloliquefaciens within inserts ranging from 5 to 15 kb. We also used pMX39 for shotgun cloning of B. subtilis α-amylase and lysine biosynthesis genes. All the genes were cloned within 5 to 30 kb inserts.

The experiments described above demonstrated that a treatment of pMX39 with BamI and ligase under conditions favoring the formation of oligomers increased the plasmid-transforming activity by nearly 2 orders of magnitude as compared to the native preparation. This is in accord with our results on the transforming properties of the pMX30 monomer (Sec. 4), which transforms B. subtilis recE4 cells 10 times worse than does the unfractionated preparation (Tab. 6). Consequently, while long inverted repeats do impart transforming properties to a plasmid, its oligomerization enhances these properties further still. We believe that the presence of inverted repeats in plasmid DNA may determine a specific mechanism of B. subtilis transformation with plasmid monomers as well as oligomers.

Section 7. Models of B. subtilis Transformation by Plasmid DNA

Schemes of transformation are depicted in Fig. 4. In regard to plasmids without inverted repeats, the schemes are in accordance with the models proposed by Canosi and coworkers (23), Michel and coworkers (25), and by other investigators (41,46). The process of transformation is arbitrarily divided into 4 stages: linearization of the plasmid molecule, penetration of a single-stranded DNA into the cell, recyclization of this strand, and duplication of the strand. Although linearization may occur at random along the plasmid, for monomer transformation it is essential that it occur within the repeat region. This is shown on the scheme. For oligomer transformation, the site of linearization is unimportant since any DNA sequence in an oligomer is a repeat.

The key stage in the model is recyclization which is accomplished through synapsis between the ends of the penetrated molecule

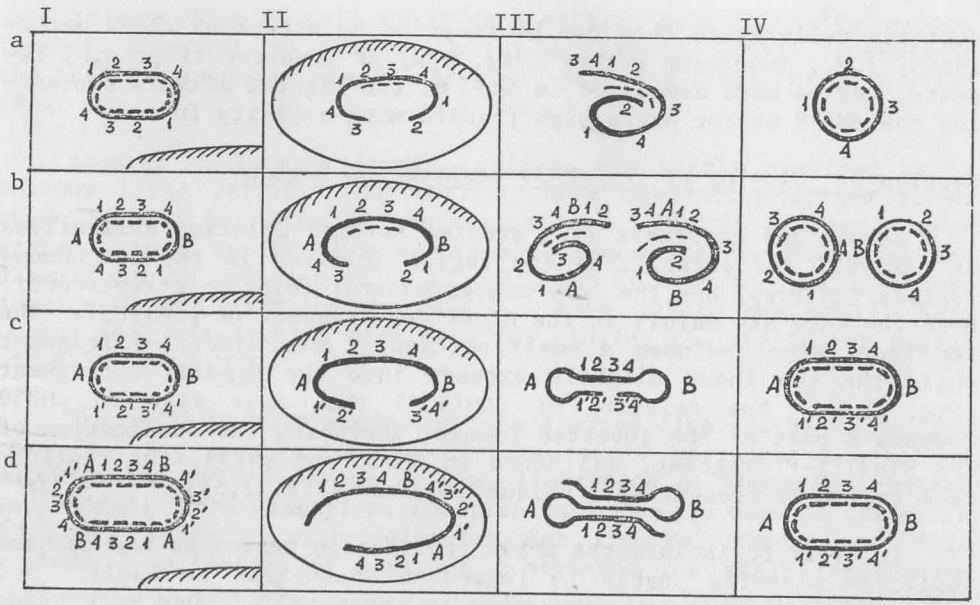

Fig. 4. Models for B. subtilis cells transformation. a) - plasmid as a dimer; b) - plasmid containing a direct repeat: c) - plasmid containing an inverted repeat; d) dimer of the plasmid with an inverted repeat. Transformation stages: I - DNA adsorbtion; II - DNA entry into cell; III- synapsis; IV - completion of transformation. Designation on DNA: repeated regions marked by numbers: 1,2,3,4, and 1',2'3',4'; unique DNA regions marked by letters; A,B.

and its inner site of homology. As can be seen from Fig. 4 (A,B), in transformation by an oligomer or by a monomer with direct repeats, the synapsis region is a triple-stranded structure. Two strands of this structure belong to 1 strand of the plasmid, and the third one, to the complementary strand. Since the plasmid as well as chromosomal DNA possibly penetrate into the cell as a single strand (11), the origin of the third strand remains a matter of speculation. It is hypothesized that small fragments of the complementary strand may "contaminate" the penetrating strand or be synthesized on it in the cell de novo (46).

The synapsis in case of plasmids carrying inverted repeats does not require any complementary DNA strand (Fig. 4:C,D). The synapsis structure may be double- or triple-stranded, and in both cases it is formed by a single DNA strand.

As is implied by the model, oligomers of a plasmid containing long inverted repeats have the best chance of transforming B.

subtilis cells since they can be recycled by either of the 2 mechanisms, i.e., involving direct (Fig. 4,A) or inverted (Fig. 4,D) repeats. As we have discussed in Sec. 6, the ligated mixture containing the pMX39 vector has a high transforming activity indeed.

Section 8. Cloning of the pMX39 Replication Region

Behnke and coworkers (47) studied several deletion derivatives of pSM19035 and established the region involved in the autonomous replication (rep) and the copy-number control (cop) in streptococci. Both the loci are mainly in the HindIII-F fragment of pSM19035. The rep locus also includes a small portion of the HindIII-B fragment while the cop locus slightly extends into the HindIII-G fragment (Fig. 2,5). The deletion of pSM19035 that gave rise to pMX30 touched a part of the inverted repeat, including the duplication of the HindIII-F fragment, and hence in pMX30 and pMX39, the replication region is present as a unique sequence (Fig. 2,5).

In order to isolate the pMX39 replication region we had to have auxiliary plasmids unable to reproduce themselves in bacilli but containing determinants expressible in these cells. One such plasmid was obtained by introducing the Em^r determinant of pMX39 into pBR322. This determinant is within the HindIII-D fragment (47). A mixture of pMX39 and pBR322 was treated with HindIII, ligated, and

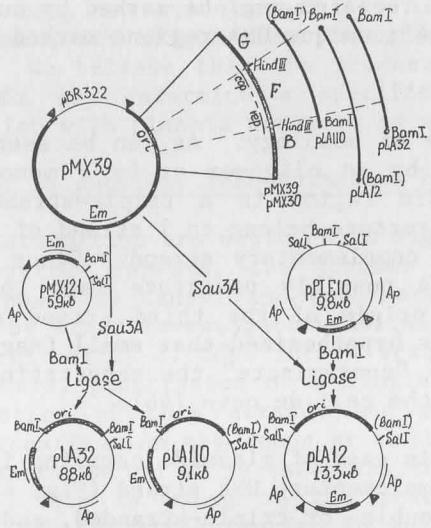

Fig. 5. Plasmids containing replication region of pMX30. (———) - pBR322; (▬) - pMX30; (▼) - EcoRI sites; The cop and rep determinants are indicated according to D. Behnke and S. Klaus (47).

used to transform E. coli rib82 recA cells. From the Ap^rEm^r transformants obtained, we isolated a 5.5-kb plasmid, pMX121, containing the pBR322 vector and the Em^r fragment joined by their HindIII sites (Fig. 5).

Another auxiliary plasmid, pPIE10, was kindly provided by A. Boitsov. The pPIE10, like pMX121, is a derivative of pBR322 at whose HindIII site the Em^r determinant of pSM22095 has been integrated. As a result of a spontaneous genome rearrangement of the plasmid during propagation in E. coli, the bulk of the pBR322 became duplicated and inverted in pPIE10. Both pPIE10 and pMX121 have unique BamI sites which were used for cloning the replicator region of pMX39.

The pMX39 DNA was subjected to a limited cleavage by Sau3A, and the resulting preparation was divided into 2 portions, to each of which either pPIE10 or pMX121 DNA, linearized at their BamI sites, were added. After ligation, the preparations were used to transform B. subtilis recE4 with selection for the Em^r phenotype. This yielded a collection of plasmids containing the pMX39 replication region within DNA fragments of varied length. Three of these plasmids having the smallest size of the cloned fragment were taken for further investigation. The plasmids were designated pLA12 (a pPIE10 derivative), pLA32 and pLA110 (pMX121 derivatives). The joining of the Sau3A and BamI cohesive ends during formation of these plasmids completely or partially restored the BamI sites at the insert ends. The DNA of pLA32 has 2 BamI sites, and pLA12 and pLA110 each have 1 BamI site (Fig. 5.). The restriction analysis of the 3 plasmids enabled us to measure the length of the "replicator" Sau3A fragments as 3.3, 2.7 and 3.0 kb for pLA12, pLA32 and pLA110, respectively, as well as to find out their coordinates on the pMX39 map. All the 3 fragments turned out to contain the rep locus, and the pLA110 fragment, in addition, contained the cop locus (Fig. 5.)

Section 9. Measuring Plasmid Copy Number

The plasmid copy number was determined as described (48) after growing plasmid-harboring B. subtilis under selective conditions at 37°C to $5 \cdot 10^8$ cells/ml. As follows from Tab. 6, pMX39 is maintained at 3 copies per genome. The reduction of the replicon size that accompanies the change from pMX39 to pLA110 entails a proportional growth of the copy number. The Sau3A fragments of pMX39 are similar to one another, as is suggested by properties of the respective plasmids. Indeed, pLA12 and pLA32 which lack the cop locus (Tab. 6 and Fig. 5) have the same copy number as pLA110 which carries the locus. This may indicate that the cop locus, which controls the copy number of pSM19035 derivatives in streptococci (Behnke et al. 47), has little if any effect on copy number determination in B. subtilis.

Section 10. Transformation of B. subtilis by a pLA12 Monomer

Electron microscopy and restriction data indicate that pLA12 carries an inverted repeat measuring 5 kb and comprising 40% of the plasmid genome (Fig. 5). This repeat is a duplication of the pBR322 segment (see Sec. 8), i.e., has an entirely different origin than the inverted repeat of pMX30. Therefore, it seemed important to find out whether the pLA12 repeat can provide for transforming activity of the plasmid monomer in B. subtilis. We isolated the pLA12 monomer by means of preparative electrophoresis and tested its transforming activity with rec^+ and rec^- cells of B. subtilis. As Tab. 7 shows, the monomer of pLA12 is an effective transforming agent. Using a double digestion with BamI and SalGI, we excised the replicator-containing fragment from pLA12 and recloned it on pMX121 (Fig. 6). The resulting plasmid, pLA1718, has the same replicator and the same Em^r determinant as pLA12, but lacks the latter's inverted repeat. As is evident from Tab. 7, the pLA1718 monomer is unable to transform B. subtilis cells. This result confirms our hypothesis on the role of inverted repeats in B. subtilis transformation (Sec. 7) and demonstrates that the transformation mechanism is essentially independent of the origin of the repeat.

Section 11. Vector Properties of pLA110

The plasmid pLA110 is a copy-number derivative of pMX39 and has some unique restriction endonuclease sites suitable for cloning (Fig. 5). We were interested in whether this plasmid retained the replicative property of its parent pMX30, namely, the ability to sustain replication of large DNA fragments. As a probe to check this, we have chosen the Rib fragment of pLP95 (see Sec. 1). The cloning scheme was identical to the one discussed in Sec. 5 (Fig. 3). The DNA of pLP95 was digested with EcoRI and joined with the pLA110 vector linearized with the same enzyme. After ligation, the preparation was used to transform B. subtilis ribD107 recE4 with

Tab. 7. Effectiveness of B. subtilis transformation by unfractionated and monomer forms of pLA12 and pLA1718 plasmid DNAs.

Form of plasmid DNA	Number of Em^r transformants in B. subtilis PG260 (recE4) per μg DNA
pLA12 unfractionated	$9 \cdot 10^3$
pLA12 monomer	$2 \cdot 10^3$
pLA1718 unfractionated	$5 \cdot 10^3$
pLA1718 monomer	0

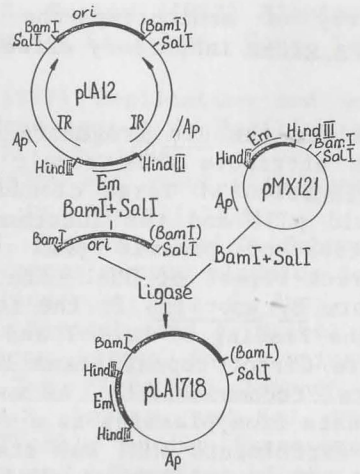

Fig. 6. Construction of plasmid pLA1718. (————) - pBR322; (■■■■) - pMX30; (⟶) - inverted repeats.

subsequent selection for $Em^r Rib^+$ markers. The plasmid pLA44 (pLA110-Rib) isolated from the transformants contained the vector and the Rib fragment joined at their EcoRI sites. The plasmid is stably maintained in B. subtilis grown in presence of erythromycin at 15 copies per genome, i.e., its copy number is greater than those of plasmids pMX33 and pMX45 constructed on the basis of the pMX30 vector (Tab. 6). It must be concluded, therefore, that the ability of pMX30 to propagate large DNA fragments in B. subtilis is due to its replication region and is retained in the pLA110 vector.

When the cells are grown under non-selective conditions, in the absence of erythromycin, pLA44 is eliminated while pMX49 persists (Tab. 6). The elimination of pMX30 and pMX39 derivatives is enhanced at higher cultivation temperatures. The observed difference in stability of the plasmids may be related to the difference in copy number. On the other hand, it cannot be ruled out that the stability determined by pMX30 is due to a certain genetic element lost during construction of the pLA32 and pLA110 plasmids.

CONCLUSIONS

In this study we attempted to estimate 2 most important characteristics of the B. subtilis vectors: their transforming activity and the ability to ensure maintenance of cloned genes. The tester used to characterize the vectors was a 9.5-kb Rib fragment of B. subtilis carrying a wild-type or a derepressed rib operon. The relatively large size of the fragment allows us to estimate the "strength" of a vector replicon, while expression of the rib genes

offers a convenient way of monitoring the fate of recombinant plasmids without causing gross inhibitory effects in the vector-host system.

Our attempts to clone the Rib fragments on the pUB110 vector were unsuccessful. We attribute this result to the inability of pUB110 to sustain replication of large cloned fragments. Cloning with the aid of plasmid pJJ1 and the intermediate host, E. coli, resulted in construction of plasmid pMX1 which contained the Rib fragment and a direct repeat of DNA. The plasmid demonstrated the ability to transform B. subtilis in the form of monomer, which is in agreement with the finding of Michel and coworkers (25). The cleavage of pMX1 at its direct repeat seems to bear a relation to the mechanism of general recombination. As was shown for E. coli, excision of direct repeats from plasmids is a recA-dependent process (50). Indeed, in our experiments pMX1 was stably maintained in E. coli rib82 recA cells. The breakdown of pMX1 in the course of B. subtilis transformation proceeds quite effectively. At any rate, our method used to detect pMX9 in the transformants failed to reveal pMX1 in them.

An investigation of plasmid pMX30 implied that long inverted repeats ensure the ability of monomers of the plasmid to transform B. subtilis. As is discussed in Sec. 10, this ability is independent of the origin of the repeats.

Cloning of Rib fragments on pMX30 demonstrated a fundamental difference between this vector and pUB110, namely, its ability to provide for stable maintenance of large cloned DNA fragments in B. subtilis. On the basis of pMX30, the pMX39 plasmid was constructed. It contains a number of restriction sites suitable for cloning, and a ligated mixture containing pMX39 possesses a high transforming activity which is essential for shotgun experiments. In our opinion, this property of pMX39 owes to the presence in it of inverted repeats.

Construction of pMX39 derivatives on the basis of the plasmid's replication region yielded copy-number variants which preserved the ability to replicate large DNA inserts. The plasmids may prove useful in the construction of vectors for gene expression. Further studies of pMX39 derivatives should specify the role of various structural elements of the plasmid in its replication and maintenance in bacilli.

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SHUTTLE VECTOR FOR ESCHERICHIA COLI, PSEUDOMONAS PUTIDA, AND PSEUDOMONAS AERUGINOSA

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SUMMARY

A hybrid plasmid capable of replication in 2 different genera, Escherichia and Pseudomonas, was constructed. This plasmid DNA can be used as a cloning vector in E. coli and pseudomonades cells.

The described hybrid plasmid pLD411 has been constructed on the basis of 2 small E. coli vector R-plasmids used in our laboratory and cryptic plasmid pWW2 or P. putida MT1. Plasmid pLD411 DNA was mapped with restrictases; its biological activity in transformations of different bacterial strains was studied, and the characteristics of transformed cells were also described.

INTRODUCTION

We could hardly be mistaken to state that more than 90% of all bacterial plasmid vector systems constructed by this time are proposed for E. coli as the most studied representative of Procariotae. As for Pseudomonas, the situation is different, and only some vector systems based on natural or in vitro constructed plasmid DNA are known now (1,2,3).

While studying some wild-type E. coli strains with plasmids that have drug resistance, we found polyplasmid strains of E. coli. By means of serial sequence steps of E. coli transformation by plasmid DNA from these strains and by selection of the clones obtained,

we succeeded in isolating 2 small plasmids determining the ampicillin- or streptomycin-resistance of E. coli (4). These multicopy plasmids, as well as hybrid structures constructed on their bases, have high transformation activity of different E. coli strains but are incapable of transforming Pseudomonas strains. In this respect they differ from RP1(RP4) or RSF1010 plasmids which can exist in E. coli and Pseudomonas strains with the same progress probably due to the peculiarities of their transcription regulation (5).

Using a cryptic plasmid of P. putida and a plasmid of E. coli, we attempted to construct a hybrid plasmid capable of replication in these bacterial cells.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The strains and plasmids used in this study are listed in Tab. 1.

Isolation of Plasmid DNA, Restriction, and Ligation of its Fragments; Determination of Contour Length, Electrophoresis, and Transformation

DNA plasmid was isolated as described in Ref. 6; cleavage by specific endonucleases and ligation of plasmid DNA fragments was carried out as described in Ref. 7; determination of contour length of plasmid DNAs was made according to procedures found in Ref. 8; the transformation of E. coli cells was carried out as described in Ref. 9; transformation of P. putida and P. aeruginosa was performed according to Ref. 10. The technique used for rapid plasmid detection in transformed clones was conducted according to Ref. 11.

RESULTS AND DISCUSSION

One of the small nontransmissive R-plasmids, isolated from a wild polyplasmid E. coli strain, was used in our study. This plasmid, designated as pLD201 of 6.14 kb, conferred E. coli strains to grow on streptomycin-containing medium (4). For our purposes, this plasmid was minimized with EcoRV restrictase and the variant obtained was designated as pLD401. The pLD401 DNA of 3.82 kb had a limited number of restriction sites for some restrictases (see Fig. 1) and it is not cleaved by BamHI, BglII, HindIII, KpnI, or SalI endonucleases. While maintaining all other characteristics inherent in parental plasmids, pLD401, with the loss of the 2.32-kb EcoRV-fragment, obtained the valuable special feature of existing in cells in more copies increased from 10 to 80 per cell. This fact influenced the level of plasmid DNA yield during its preparative isolation. One must note that the addition of chloramphenicol in the

Tab. 1. Bacterial strains and plasmids.

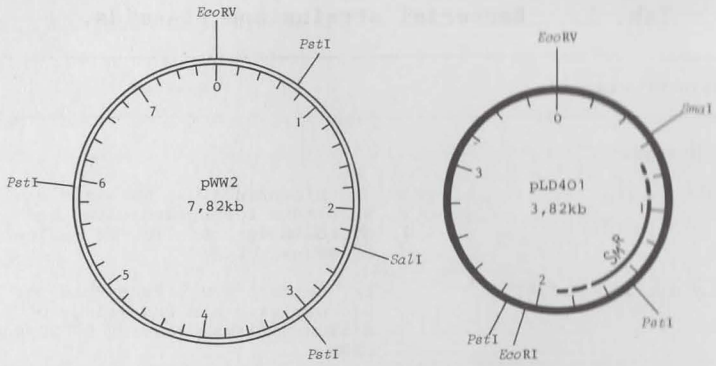
Strain or plasmid	Source*
Strains	
E.coli C	Dr. Aleshkin A.G., Gamaleja's Institute for Epidemiology and Microbiology, Academy of Medical Sciences, USSR
P.aeruginosa PAO ML4262	Dr. Boronin A.M., Institute for Biochemistry and Physiology of Microorganisms, Academy of Sciences, USSR
P.aeruginosa PAO ML4600	same
P.putida BS250	same
P.putida mt-2 PaW85	Dr. Heinaru A.L., Tartu State University, Estonian SSR, USSR
P.putida MT1	same
P.putida MT1(Tol$^-$)	see text
Plasmids	
RSF1010	Dr. Boronin A.M., see above
pLD201	see text
pLD401	see text
pLD411	see text
pWW2	see text

* - Bacterial strains obtained from Dr. Heinaru A.L. while collaborating on biodegradated plasmids.

nutrient medium did not result in an increase of copy-number of the plasmids described, and their DNAs could not transform P. putida or P. aeruginosa strains.

Plasmid pWW2 of P. putida MT1 (12) of molecular mass 4.8±0.1 Md was another plasmid used in our study. This plasmid DNA was spontaneously isolated from the MT1 strain with the loss of another plasmid pWW1 of 52.0±0.4 Md in nonselective conditions of cultivation (L-broth). With the loss of plasmid pWW1, the strain cells lost their ability to use toluate as the only source of carbon. The properties of the strain (Tol$^-$) were similar to those of P. putida MT1-B1 isolated during the growing of parental strain MT1 on benzoate (13).

The study of pWW2 DNA showed that it had the only unique restriction site for EcoRV and SalI, 3 sites for PstI, but was not cleaved by EcoRI, BamHI, HindIII, and BglII endonucleases.



EcoRV & T_4-DNA LYGASE STEPS

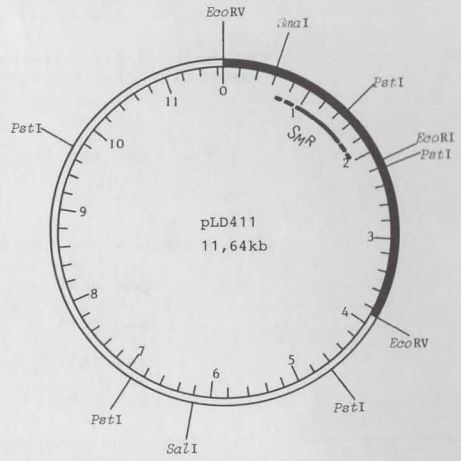


Fig. 1. Construction of the pLD411 plasmid on the basis of pLD401 and pWW2 plasmids. Plasmid pLD401 and pWW2 DNA were mixed in equimolar concentrations in restriction buffer containing 100 mM Tris·HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$. Then the required quantity of EcoRV was added and the reaction maintained at 37°C for 60 min. DNA fragments were subjected to heating up to 65°C for 10 min and dialyzed with 66 mM Tris·HCl (pH 7.6), 6.6 mM $MgCl_2$. DTE and ATP were added with concentrations of 10 and 0.5 mM accordingly with required quantity of T_4-DNA ligase, and the reaction maintained at 4°C for 16-18 hr.

To obtain hybrid plasmid molecules, pLD401 and pWW2, their DNAs were mixed in equimolar quantities, cleaved by EcoRV endonuclease and the fragments obtained were ligated by T_4-DNA ligase. The E. coli C strain was transformed by the mixture of ligated fragments. The clones carrying hybrid plasmids were selected among the transformants resistant to 100 µg/ml of streptomycin using the technique

Tab. 2. Frequency of transformation (cells per µg DNA).

Plasmids	Bacterial strains				
	E.coli C	*P.putida*		*P.aeruginosa* PAO ML	
		BS250	mt-2 PaW85	4262	4600
pLD401	1.3×10^4	0	0	0	0
pLD411	1.0×10^3	4.7×10^2	5.8×10^2	1.1×10^3	1.3×10^3
RSF1010	2.2×10^4	3.3×10^2	7.1×10^2	1.8×10^3	2.1×10^3

described in Ref. 11. The clones of E. coli C, P. putida mt-2 PaW85 and BS250, and P. aeruginosa PAO ML 4262 and 4600 were transformed by hybrid plasmid DNA isolated from those clones and designated as pLD411. Plasmid RSF1010 DNA was used as a positive control and pLD401 as a negative one. The results of transformation are presented in Tab. 2, and the general scheme of obtaining of the hybrid plasmid pLD411 and its restriction map are shown in Fig. 1. Table 2 shows that plasmid pLD411 DNA transforms E. coli C as well as both Pseudomonas species. Moreover, in both cases the transformation frequency could be compared to that when plasmid RSF1010 DNA was used. The presence of the integral hybrid structure in transformed cells of different strains was confirmed by using the technique described in Ref. 11; plasmid DNAs of those strains we analyzed by means of restrictases and the data obtained enabled us to make a restriction map of pLD411.

The maximum streptomycin-resistance level of transformed E. coli strains was 1 mg/ml and the level of both species of Pseudomonas was more than 2 mg/ml of medium. The strain containing hybrid plasmid maintained streptomycin-resistance in nonselective conditions during 12 passages; the plasmid was not undergoing any structural alteration in this situation.

Plasmid pLD411 DNA can be used as a bifunctional vector in suitable Escherichia or Pseudomonas strains inserting the necessary genetic material in unique restriction sites. We believe that the plasmid functioning in cells of different genera was connected with separate use of its 2 parts for full replication of the whole structure. With regard to this fact, the plasmid is similar to bifunctional vector molecules used for E. coli and Saccharomyces yeast.

ACKNOWLEDGMENTS

We are thankful to S.V. Solovjeva for her excellent contribution in the construction and mapping of pLD401 vector DNA.

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REPORT ON A WORKSHOP:

PLASMID CLONING VEHICLES

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The first three contributions addressed the problem of designing plasmid vectors for the expression of foreign proteins in E. coli. All three used the principle that, to reduce the genetic and metabolic load to the cell, low or moderate copy-number plasmids should be used, and in two cases higher copy numbers were induced late in the growth phase. Yarraiton (Celltech) reported on experiments to express calf met-prochymosin. Initial experiments with the high copy ampicillin-resistance plasmid (based on pAT153) using a trp promoter and T7 terminator gave 5% loss/generation which could not be stabilized by ampicillin selection during scale-up. Repressed levels of production even in the presence of the pSC101 par locus caused instability because the trp promoter was not fully repressed when it was on the high copy plasmid. Vectors were therefore constructed in which the primer RNAII transcription could be brought under control of various inducible promoters, in particular, bacteriophage P_L under the control of the λcI857 gene product. In the presence of the pSC101 par region these plasmids carrying the trp-controlled prochymosin gene were stable at 30°C in the absence of antibiotic selection, having a copy number of 5/genome. This stability was high enough to meet the stringent industrial requirements with maintenance of excellent cell growth properties (final O.D.=60). On induction (37°C), a copy number of 400/cell was reached in which 10 to 5% of total protein was prochymosin. For the production of Insulin A and B chains (as β-galactosidase-Insulin A/B are trp E-Insulin A/B fusions) high expression from the lac or trp promoters on high copy plasmids gave 50% loss by the twentieth generation (Hershberger, Eli Lilly and Company). Using a down copy pBR322 derivative and transcriptional fusion of the expressed gene and Tc^R combined with the presence of the CI_{857} repressor on the plasmid in a CI^- lysogenic strain (i.e., suppression of a lethal

[1] Eli Lilly and Company, Indianapolis, Indiana.

phenotype forces plasmid maintenance), stability was achieved and at the end of production the cells could be lysed by lytic induction at 42°C for product release. For another interesting presentation on the use of fused peptides for gene product formation see Bastia (this volume), in which a collagenase-sensitive peptide is used as a digestible fusion bridge allowing later release of the mature peptide.

A third vector system which should have similar properties to those described above is based on the naturally low copy plasmid CloDF13 (Van Putten, Free University, Amsterdam) and uses a mutant temperature inducible for higher copy number. A hybrid was described containing the amp^r and tet^R genes of pBR322, the trp E gene and promoter and a strong downstream transcription terminator (see Bujard, this volume for the importance of strong terminators to prevent interference with replication) and the temperature-inducible mutant replicon of CloDF13.

A second main theme introduced by S. Ehrlich (Paris) and Hershberger was the formation of multimeric DNA segments (gene amplification) in the chromosomes of Bacillus subtilis and Streptomyces species. Ehrlich (this volume and Pseudomonas workshop) presented his proposal of integrating recombinant DNA more stably into the chromosome of B. subtilis ("chromosomal vectors"). The amplification of Neo^R-, Amp^R-, and Cm^R- inserts located between direct repeats could be induced for the Ery^R- but not for resistance genes. Coselection for Neo^R and Amp^R gave up to 50 copies/genome. Rownd (Northwestern University Medical School) reported that such amplification (a few copies) could be induced by antibiotic selection in a rec A ts E. coli mutant which then remained stable at the restrictive temperature (Hoffman, Rownd, in press).

Hershberger has identified a 10.5-kb region carrying an amplifiable unit of DNA and bounded by 2.2-kb direct repeats of the Streptomyces fradiae genome. Amplification of this 10.5-kb unit to give 500 tandem repeats (30% of the total final DNA) was obtained. Other naturally amplified sequences have been reported for different Streptomyces species by R. Hutter, H. Schrempf, M. Robinson, and U. Hornamann. At least 3 of the amplified DNAs are different as judged by a lack of homology detectable by hybridization.

The formation of concatamers (integrated "picolosomes": after Wigler) in the genomes of eukaryotic cells is used in the cosmid shuttle system to identify genes expressing in heterologous cells and to reisolate them in E. coli as cosmid hybrids via in vitro packaging (λ in vitro packaging system) of the crude genomic DNA from a eukaryotic cell clone (Collins, Braunschweig; see Lindenmaier, et al. 1983, Nucl. Acids Res.). It was proposed that this system might be effectively applied to any organism in which tandem repeats can be stably maintained. Recent advances in cosmid cloning methodology were reviewed in which the use of double cos

vectors can facilitate the use of an Ish-Horowitz and Berg type method to prevent this formation of multimers of the vector and the use of dephosphorylated target DNA to prevent the cloning of non-contiguous fragments in a single hybrid. The use of an in vivo repackaging system allows the maintenance of the cosmid hybrid gene bank as a lysate of packaged particles (as for λ) which ensures stable maintenance of the bank over periods of several years. The development of a recombination screening method for cosmids analogous to that developed by Brian Seed for λ the π Vx-System) was reviewed [Collins, Braunschweig (in press), Lehrach et al., (in preparation), and Lindemaier and Houser]. In this system a small plasmid having no homology to the cosmid vector carries a DNA "probe." On transduction of the cosmid library into a λ lysogenic cell carrying this hybrid, the hybrid can cointegrate into a cosmid hybrid having homology in its insert. Subsequent in vivo repackaging and selection for antibiotic resistance markers carried by the cosmid and the small "probe" plasmid allows isolation of the co-integrate. This method is an alternative to colony hybridization for the isolation of long genomic fragments homologous to the DNA probe, and has the advantage that, due to the short residence time of the cosmid hybrid in E. coli (a few hours), even relatively unstable clones (e.g., human interleukin 2 gene) can be isolated.

Two further shuttle-vector systems were presented, one by Bagdasarian (Umia) for a broad host-range cosmid based on RSF1010 [see Pseudomonas workshop and Frey et al. Gene (1983)] and by Hershberger for E. coli/Streptomyces, based on the pock genes and ORI from SCP2. One variant of the latter can give single integration events leading to small "pocks." The presence of Neo^R and $thiostrepton^R$ genes on one vector allows insertional inactivation to be used to identify hybrid molecule-containing clones.

Although the development of small modifications to the huge range of existing vector systems to meet "local needs" is within the capability of most molecular geneticists, a few radical innovations can be seen to be constantly in development. For the further rational developments of vectors a better understanding of plasmid molecular biology, DNA replication, transposition, and recombination is required. This symposium provided useful additions to our understanding of these areas.

PLASMIDS IN THE DEGRADATION OF CHLORINATED AROMATIC COMPOUNDS

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INTRODUCTION

The soil-borne microbial community takes the "lion's share" of the mineralization process of the ever-increasing input of man-made organic compounds in the biosphere, converting them to biomass or maintaining the balance of various elements in the soil (1). In the broad spectrum of stability of these compounds, halogen-substituted aromatic pollutants are of major concern because of their recalcitrance and magnitude of use (2). Haloaliphatic compounds possess shorter half-life in nature, most probably because of the presence of broad-specificity enzymes which can dehalogenate such compounds and are known to be active in Pseudomonas, Flavobacterium sp., and Moraxella sp. (3,4,5,6). In the past few years, isolation of pure cultures capable of degrading various compounds that are toxic and classified as recalcitrant, such as 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2,4-dichlorophenoxyacetic acid (2,4-D), pentachlorophenol, etc., boosted up research in both the fields of basic understanding of the metabolic pathways of these compounds as well as in their potential use in decontamination technology (7,8,9,10,11).

The present state of research increasingly strengthens the emerging concept that, like the multiple drug-resistance markers both in enteric and other pathogenic bacteria, the metabolic versatility of soil bacteria, with few exceptions, is determined totally or partly by resident plasmids (12).

On the basis of the nature of plasmid involvement in the biodegradation of haloaromatic compounds, 3 functional categorizations may be formulated.

(a) Plasmid material is mostly independent of the host because it carries at least all the structural genes necessary for the

degradation of a specific compound to simple organic acids which are subsequently incorporated into the general metabolic pool. For instance, plasmid pWR1 or pAC25 of P. putida codes for all the enzymes necessary for the conversion of 3-chlorobenzoic acid (3Cba) to maleylacetic acid (13,24), but in these cases, host-coded enzymes can also complement part of the functional machinery because of the presence of an extra set of chromosomally coded enzymes (13,14).

(b) In the second category, some of the chromosomally coded enzymes, because of their broad specificity, can attack both halogenated and nonhalogenated substrates, leading to dead-end products like halocatechols in case of haloaromatic compounds (15,16). This block in the catabolic pathway is removed by the plasmid-encoded enzymes which have high specificities towards halogenated substrates (13,14,17,18,19). This case is best exemplified by Alcaligenes eutrophus containing plasmid pJP4 which can catabolize chlorocatechols, the dead-end products of 3-chlorobenzoate degradation, by the 2-step, chromosomally coded enzymes, benzoate-1-2 dioxygenase and dihydrodihydroxybenzoate dehydrogenase. Therefore, dissimulation of a haloaromatic compound is carried out by joint action of host and plasmid (13,14).

(c) In the third category, presence of side chain on the halogen-substituted aromatic ring makes them difficult substrates to degrade for host enzymes. The general assumption that cleavage of the side chain in 2,4-D and 4-chlorophenoxyacetic acid (4Cpa) is a plasmid-coded function (9,14) has not been challenged. For example, in case of 2,4-D, hydrolytic cleavage of the ether bond to remove the side chain, thus converting this compound to the corresponding chlorophenol, is believed to be a plasmid-specified function. The chlorophenol formed could then be degraded either by plasmid- or host-coded enzymes (9). A. paradoxus, containing plasmid pJP1 capable of degrading 2,4-D, is a well-known example of such a mechanism (9).

Knackmuss and his associates worked out the metabolic pathway for degradation of chlorobenzoates by Pseudomonas sp. B13 (19,20), identified the enzymes involved, and described the range of specificity of enzymes encoded by either chromosome or plasmid (20,21). Weightman et al., (14) recently proposed converging pathways for monochlorobenzoate and chlorophenoxyacetic acid by A. eutrophus harboring pJP4 via chlorocatechol. In order to study the role of plasmid and host determinants in the degradation of haloaromatic compounds, we have selected 2 of these well-investigated compounds, 3Cba and 2,4-D, and another compound, 2,4,5-T, which is least understood in terms of both catabolic pathway and degradative genes and enzymes involved.

Our laboratory is engaged in dealing with the following questions in relation to haloaromatic catabolism:

(a) The minimum segment of the plasmid DNA necessary for efficient expression of 3Cba$^+$ or 2,4-D$^+$ phenotype.

(b) Localization of the catabolic genes on the physical map of a catabolic plasmid.

(c) The nature of regulation or expression of a catabolic pathway and the regulatory genes involved.

(d) Do genes encoding similar catabolic steps but borne on different plasmids share any structural and functional homology as reminiscent of their common evolutionary origin?

(e) Is the evolution or spread of these catabolic genes similar to that of the multiple drug-resistance genes carried by various drug-resistance plasmids?

(f) To what extent are various legitimate and illegitimate recombinational events, such as amplification, deletion-fusion, insertion etc., which are basic mechanisms in evolution, operational in the evolution and dissemination of such catabolic plasmids?

(g) Do these catabolic plasmids possess unique structural and genetic features that provide them with raw materials for a unique mode of regulation and rapid evolution with dramatic increase in their catabolic voracity in response to selection pressure?

(h) What are the genetic and biochemical bases for specific association and expression of a specific catabolic pathway shared between a host bacterium and a plasmid?

Some of the answers to the above questions are discussed in the next few sections.

Plasmid-specified Genes in the Biodegradation of 3-Chlorobenzoic Acid (3Cba)

The transmissible nature of the 3Cba phenotype, both in Pseudomonas and Alcaligenes and other biochemical studies, indicates the essential participation of plasmids in total degradation of 3Cba (13,23,24,25). All the 5 enzymes necessary for the conversion of 3Cba to maleylacetic acid were shown to be present on plasmid pWR1 (13) which has been shown to be structurally homologous to pAC25 or pAC27, except a 6-kb deletion in pWR1 (26). Unlike pAC27, Alcaligenes plasmid pJP4 has been shown to encode only 3 enzymes for chlorocatechol catabolism and these appear to be similar in function with Pseudomonas sp. B13 plasmid pWR1-coded enzymes (14). The first 2 enzymes for the conversion of 3Cba to 3-chlorocatechol could be

provided by chromosome both in Pseudomonas sp. B13 and A. eutrophus (13,14).

We have previously demonstrated the operation of the modified ortho pathway for 3Cba degradation encoded by pAC25 or pAC27. pAC27 is identical to pAC25 with regard to all known functions, but has an 11-kb segment deleted from pAC25 (27). To localize the genes for 3Cba degradation on plasmid pAC27 and pJP4, we took into consideration the fact that significant homology is expected between 2 plasmids only in the region carrying the 3Cba structural genes. Subsequent cloning of the regions in a broad host-range vector will undoubtedly localize the 3Cba genes. Physical maps for these 2 plasmids are given in Fig. 1A.

Following this rationale, EcoR1 fragments A, B, C/D, E, F, and G of pJP4 were purified, nick-translated, and used as probes for hybridization with EcoR1, HindIII, and BglII digested restriction fragments of pAC25 transferred from agarose gels onto nitrocellulose filters. Homology was detected only with EcoR1-B and BglII-E fragments of pAC27 when EcoR1 fragments B and E of pJP4 were used as probes (Fig. 1B and 1C). No hybridization was detected with any of the pAC25 fragments when EA, EC/ED, EF, and EG fragments of pJP4 were used as probes (data not shown). In an analogous experiment with BglII-E fragment (Fig. 1A) of the plasmid pAC25 was purified, nick-translated, and used for hybridization with EcoR1 and EcoR1/BamH1 digested pJP4 DNA fragments blotted to a nitrocellulose filter. As expected, only the EcoR1 fragments B and E and EcoR1/BamH1 double digested fragment C (10 kb) and F (3 kb) of pJP4 showed homology with the probe DNA fragment (Fig. 2). The region of homology between these 2 plasmids is diagrammatically presented in Fig. 1A.

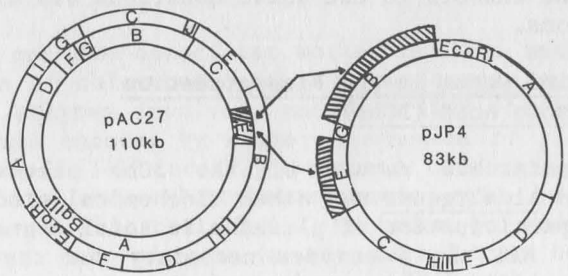

Fig. 1. A. Endonuclease cleavage map of plasmid pAC27 for EcoR1 and BglII sites and pJP4 for EcoR1 sites. Hatched areas on both the plasmids show the location of the chlorocatechol degradative genes. No homologous counterpart of EcoR1 fragment EG of pJP4 is found in pAC27. Area encompassed by 2 arrows on pAC27 (BglII E fragment) and pJP4 (EcoR1 fragment EB and EE) denote the region of homology between the 2 plasmids.

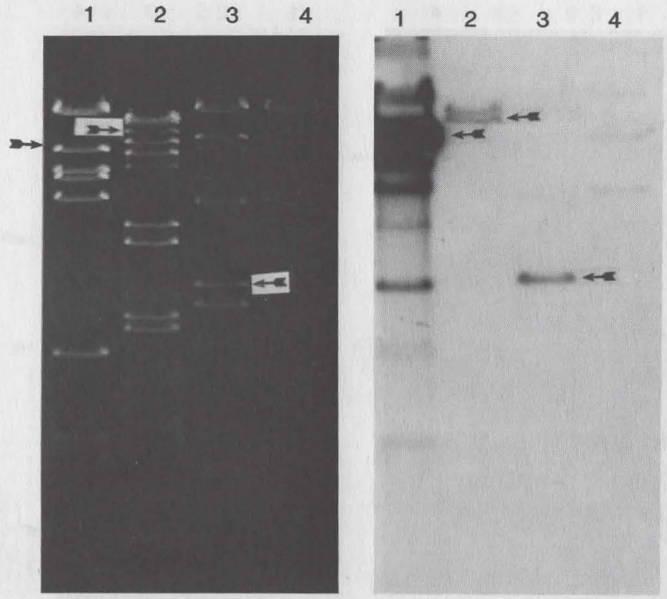

Fig. 1. B. Agarose gel electrophoresis of restriction fragments pJP4, EcoR1 (lane 1), pAC25 EcoR1 (lane 2), pAC25 BglII (lane 3), and pAC25 HindIII (lane 4). Corresponding Southern blot of the gel, using EcoR1 fragment B of pJP4 (lane 1) as a nick-translated P^{32}-labeled probe, is shown on the right. Arrow shows the specific bands hybridized to such probe.

It is clear from the hybridization data that the segment representing sequence homology between the 2 plasmids is clustered in pAC27 on the 4.2-kb BglII-E fragment, whereas, the fragment EG of plasmid pJP4 with no detectable homology to pAC27 divides this region in pJP4 into 2 segments (Fig. 1A).

Cloning of Restriction Fragments of pAC27 and pJP4

Broad host-range cosmid vector pLAFR1 (28), specifying tetracycline-resistance (Tet^r), was used to construct EcoR1 gene libraries of plasmid pAC27 and pJP4. The size of insert cloned in this phage lambda-derived vector is 20-30 kb (28). Partially EcoR1-digested plasmid DNAs were ligated to the EcoR1-cleaved vector, packaged in phage-lambda heads used for infection in Escherichia coli AC80 (Thr^- Leu^- Met^-) and selected for Tet^r. The plasmid pool was mobilized en masse from E. coli to P. putida PpG1900 (Kan^r Trp^-) or AC10 (Met^-) in a triparental mating using E. coli HB101/pRK2013 as a mobilizer (29). Conjugation mixtures were either plated on minimal glucose containing Tet or on 3Cba plates with recipient amino acid supplements. When plated on glucose Tet plate, colonies

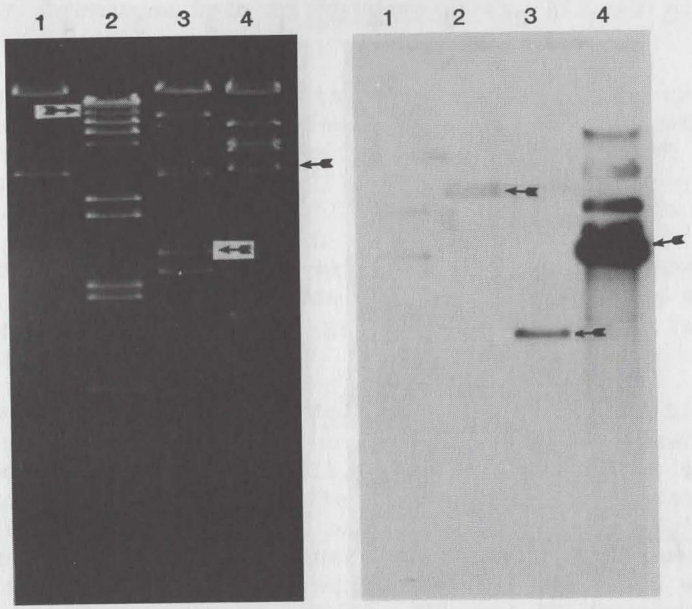

Fig. 1. C. Same as Fig. 1B; pAC25 HindIII (lane 1), pAC25 EcoR1 (lane 2), pAC25 BglII (lane 3), and pJP4 EcoR1 (lane 4). EcoR1 fragment EE (lane 4 arrow) of pJP4 was used as a probe.

from such plates were tested for their 3Cba$^+$ phenotype. Restriction analysis of such 3Cba$^+$ clones is shown in Fig. 3A and 3B.

A 3Cba$^+$ clone obtained from pAC27 revealed the presence of pAC27 EcoR1 fragment B and J only in the plasmid designated as pDC10 (Fig. 3A), whereas pJP4 derived clones such as YG410, and YG419 showed the presence of pJP4 EcoR1 B and H, and only pJP4 EcoR1 B fragment, respectively, besides pLAFR1. Unlike pDC10 which allowed rapid growth with 3Cba, the plasmids such as pYG410, pYG419, etc., present in YG410 or YG419 allowed only slow growth on 3Cba with accumulation of brown-colored products. The restriction analysis of such plasmids (e.g., pYG419) demonstrated the presence of the pJP4 EcoR1 B fragment in relatively high intensity as compared to pLAFR1 (Fig. 3A) indicating the presence of multiple copies of EcoR1-B fragment on this plasmid. Electron microscopic visualization revealed a large plasmid of about 120 kb (Fig. 4). This plasmid on denaturation and renaturation did not show any "snap back" structure suggesting that the multiple EcoR1-B fragments are presumably present in tandem array. Slow growth of this strain in 3Cba suggested the presence of all the structural genes for chlorocatechol degradation on the EcoR1-B fragment of pJP4. These genes have subsequently been located more precisely on the 10-kb BamH1/EcoR1-C fragment. It

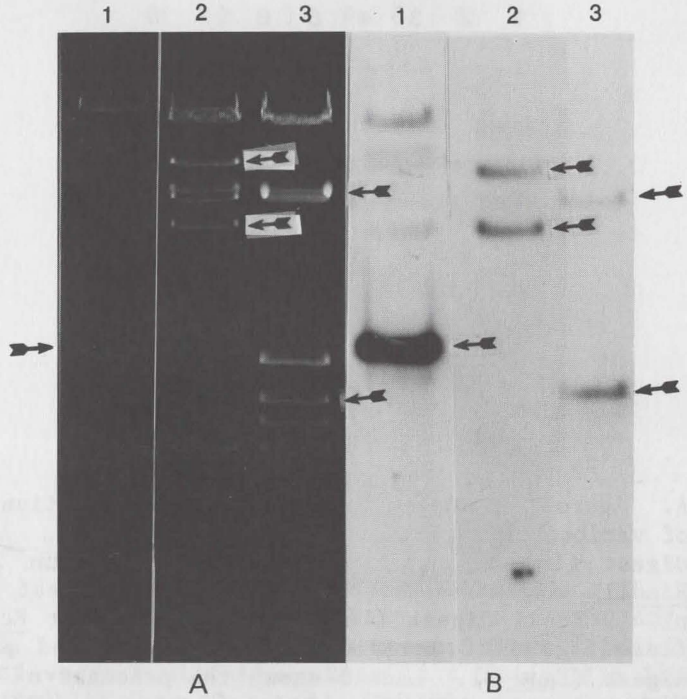

Fig. 2. Agarose gel electrophoresis (2A) of BglII digested pAC25 (lane 1), EcoR1 digested pJP4 (lane 2), and BamH1/EcoR1 digested pJP4 (lane 3). Southern blot (2B) of the same gel shows the homology of BglII E fragment (P^{32}-labeled probe) of pAC25 with EcoR1 fragments B and E of pJP4 (lane 2) and 10-kb and 3-kb segments of pJP4 (lane 3).

was, however, apparent that an additional segment of pJP4 is necessary for the efficient expression of chlorocatechol genes. Absence of this regulatory gene is partly compensated for by amplification of the segment containing the structural genes, i.e., a gene dosage effect with pYG410 and pYG419. Since amplification of the structural genes allowed slow growth in absence of the regulatory gene product, it appears that the 3Cba degradative genes on pJP4 are, presumably, positively regulated. Additional evidence for this assumption will be provided in the next section.

The EcoR1-B fragment of pAC27 cloned on pDC10 is a 20-kb segment. To further localize the chlorocatechol-degradative genes in a shorter segment, pAC27 DNA was digested with BglII and ligated to a plasmid vector pRK290 (29) and a gene bank was constructed in E. coli AC80. Screening of 3Cba$^+$ clones from this gene bank after mobilization into P. putida PpG1900 revealed the presence of 3 classes of clones (Fig. 3B). Class I contains 2 continuous BglII fragments E and C (pDC15) and shows the best phenotypic expression; Class I

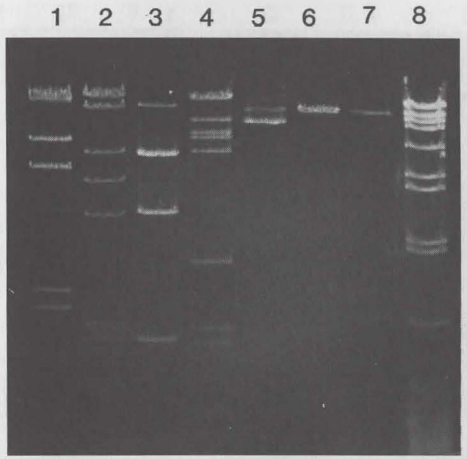

Fig. 3. A. Agarose gel electrophoresis of restriction fragments of various DNAs derived from pJP4 and pDC10. λ DNA EcoR1 digest (lane 1); pJP4 HindIII digest (lane 2); pYG419 HindIII digest (lane 3); pJP4 EcoR1 digest (lane 4); pYG419 EcoR1 digest (lane 5); pLAFR1 vector EcoR1 digest (lane 6); pDC10 EcoR1 digest (lane 7); and pAC27 EcoR1 digest (lane 8). Lane 5 shows the presence of only EcoR1 B fragment of pJP4 in multiple copies in pYG419 (higher intensity of the insert compared to vector) and lane 7 shows EcoR1 B and J fragments of pAC27 in pDC10.

contains fragments E and F (pDC20) and demonstrates poor growth ability in minimal 3Cba medium. One Class III clone (DC25) was found to contain only a BglII-E fragment (pDC25) and also grows slowly on 3Cba. These data, in combination with previous results on hybridization, clearly suggest that chlorocatechol-degradative genes are homologous in plasmids pAC27 and pJP4 and are located on BglII-E fragment (4.2 kb) of pAC27 and in a 10-kb segment (BamH1/EcoR1 fragment C) of pJP4.

To localize the putative regulatory gene(s) on plasmid pJP4, 15 individual AC80 Tet^r clones from the E. coli gene bank were randomly screened for the composition of recombinant plasmids by miniscale plasmid preparation (30). Seven clones all of which contain the pJP4 EcoR1-B fragment plus one or 2 of the EC, ED, EE, EF, EG, and EH fragments were selected, the plasmid from each clone was individually mobilized into various P. putida strains and tested for their $3Cba^+$ phenotype. Two $3Cba^+$ clones (Y43, Y52) were obtained from such transfer. Both of them grew well in minimal 3Cba medium, and restriction analysis of the plasmids from these 2 strains, grown in 3Cba-medium, showed them to be identical, i.e., both contained plasmids with EcoR1 fragments EB, EE, and EF (Fig. 3B). In subsequent

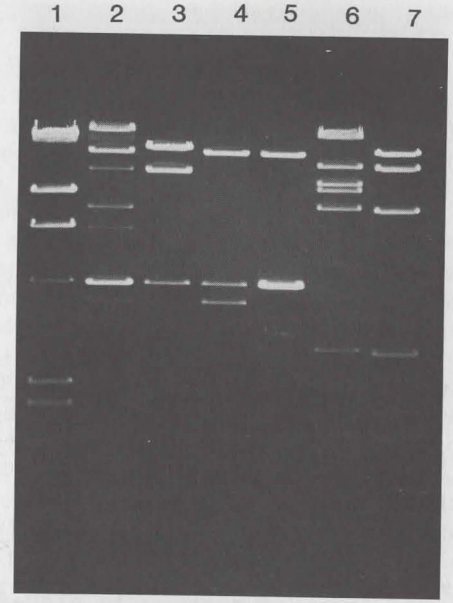

Fig. 3. B. Agarose gel electrophoresis of restriction fragments of various plasmids. Lane 1, λ (HindIII); lane 2, pAC27 (BglII); lane 3, pDC15 (BglII); lane 4, pDC20 (BglII); lane 5, pDC25 (BglII); lane 6, pJP4 (EcoR1); and lane 7, pY43 (EcoR1).

transformation experiments, this plasmid pY43 also conferred Tet^r $3Cba^+$ phenotype to various P. putida strains. Since EB alone or combinations of EB and other fragments besides EE and EF did not produce $3Cba^+$ phenotype readily, it appears that fragment EE and possibly EF are essential for rapid growth with 3Cba. The homology of the pJP4 EE fragment with the pAC25 BglII-E fragment (Fig. 2) further indicates that the EE fragment may contain the regulatory gene(s) needed for efficient expression of the chlorocatechol-degradative genes present on the pJP4 EB fragment.

Structural and Functional Features of the Plasmids Involved in Chlorocatechol Degradation

It is safe to assume from the results already presented that though the structural genes for chlorocatechol degradation are clustered and apparently homologous in both pAC27 and pJP4, some interesting structural and functional features indicate their remarkable evolutionary divergence.

Functionally, the plasmid pDC25 harboring the pAC27 4.2-kb BglII-E fragment or pDC10 containing the EcoR1-B fragment of pAC27

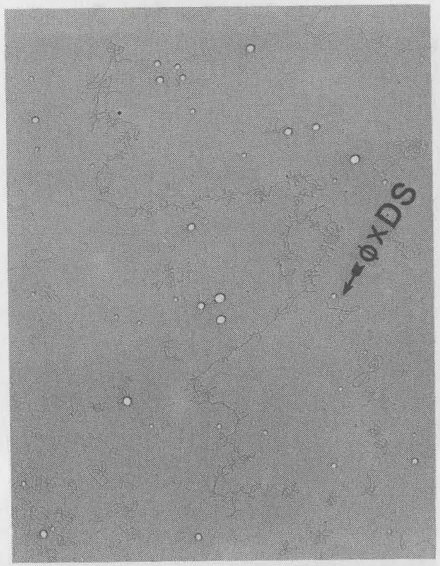

Fig. 4. Electron micrograph of covalently closed circular molecule of pYG419.

when transferred to various strains of P. putida or P. aeruginosa readily expresses the 3Cba$^+$ phenotype. The plasmid pJP4 belongs to the P1 incompatibility group and can be transferred from its original host (A. eutrophus) to P. putida at a frequency of 10^{-2} to 10^{-3} when selected on PIA (Pseudomonas Isolation Agar) medium containing merbromin (100 µg/ml) or mercuric chloride (40-50 µg/ml); however, the 3Cba$^+$ phenotype is recovered from the recipients with a frequency of 10^{-6} or less. Intact plasmid pJP4 was recovered from 3Cba$^-$ Hg$^+$ P. putida exconjugants (You and Ghosal, unpub. results). It has not been possible to grow P. putida harboring pJP4 in liquid minimal 2,4-D medium, though poor growth could be seen on 2,4-D plates.

Plasmid pJP4, like other 2,4-D-degradative plasmids such as pJP1, pJP2, etc., was originally isolated from Alcaligenes sp., whereas both pAC25 and pWR1 were originally isolated from Pseudomonas sp. A pertinent question is whether the difference in expression is due to an inherent variation in the structural and regulatory genes on the 2 plasmids or due to a mismatch of the signal sequences present on plasmid pJP4 with the transcription-translation machinery of the new host, i.e., Pseudomonas sp. The second explanation is plausible since Pseudomonas plasmids are known to be expressed poorly in other hosts, particularly the enteric bacteria. The presence of unique structural features present in the plasmid pJP4, however, appears to indicate that a variation in the continuity of the structural and regulatory genes pertinent to 3Cba degradation is the major difference between pAC25 and pJP4. The EcoR1-E fragment (EE) of pJP4 which demonstrates homology with pAC25 BglII-E fragment possesses a unique structural feature, a "stem-loop" structure (1.8-kb stem and 1-kb loop) not present in pAC25 DNA (Fig. 5). It has already been mentioned that the presence of intact plasmid pJP4 in P. putida does not confer 3Cba$^+$ phenotype readily. Continued selection does allow emergence of 3Cba$^+$ colonies. Plasmid DNA isolated from such 3Cba$^+$ exconjugants were found to show unique structural rearrangements involving EcoR1 fragment E (EE) in the region of the above mentioned "step-loop" structure and most likely fragment B (EB) and F (EF). The type of rearrangements was found to be identical in the plasmid profiles isolated from 5 independent isolates of 3Cba$^+$ P. putida. A typical plasmid with such rearrangement was designated as pYG3, which on EcoR1 digestion produced only 5 fragments instead of 10 for pJP4 (Fig. 6). Alcaligenes eutrophus harboring pJP4 (JMP134) on being maintained in 2,4-D media for many generations does not express 3Cba$^+$ phenotype readily. Similar to P. putida, 3Cba$^+$ Alcaligenes colonies can be obtained after prolonged selection on 3Cba plates. Restriction analysis of the plasmid DNA isolated from 3Cba grown Alcaligenes sp. demonstrated the presence of both pJP4 as well as the pYG3 type of plasmids with similar genetic rearrangements (Fig. 6). The structural changes of plasmid pJP4 which resulted in pYG3 forms were investigated by electron microscopic analysis, restriction enzyme digestion, and Southern hybridization. All 5 EcoR1 fragments of pYG3 were purified and separately

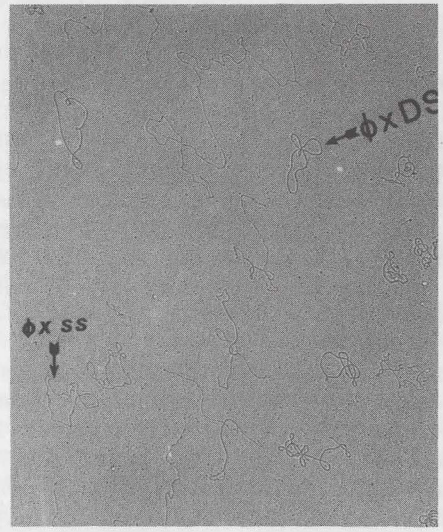

Fig. 5. Electron micrograph of "snap back" structure of EcoR1 fragment EE of pJP4 showing a 1.8-kb stem and a 1-kb loop.

Fig. 6. Agarose gel electrophoresis of λ digested with HindIII (lane 1); EcoR1 digested pYG3 (lane 2), and pJP4 DNA isolated from 3Cba-grown Alcaligenes eutrophus (lane 3) showing deletion. Lane 3 demonstrates the presence of 2 plasmids (both pJP4 and pYG3); pJP4 digested with EcoR1 (lane 4).

used as probes for hybridization with EcoR1 fragments of pJP4. Such hybridization results demonstrated that in pYG3, a deletion of approximately 15-kb DNA occurred with loss of pJP4 EcoR1 fragments, EC, EH, EI, and 1.6-kb portions of fragment EE, and unknown amounts of EF and EG. Such deletion events created 3 new fragments, pYG3 EA' is the same as pJP4 EA, pYG3 EB' is the same as pJP4 EB, pYG3 EC' is derived from pJP4 fragments ED and EE, pYG3 ED' is derived from pJP4 fragment EF while pYG3 EE' is a very short deletion derivative of fragment EG.

Although restriction and hybridization data indicated the complete or partial loss of several fragments from pJP4 to generate the plasmid pYG3, electron microscopic contour-length measurement demonstrated the size of pYG3 of the order of 93 kb, compared to 83 kb of pJP4. An inverted duplication of a 25-kb segment involving EcoR1 fragment E (EE) and G (EG) and most likely part of B and F occurred in pYG3. Such molecules of pYG3 on denaturation and fast renaturation give rise to a long-stem loop structure with a 25-kb stem and 1.3-kb and 43-kb loops as shown in Fig. 7.

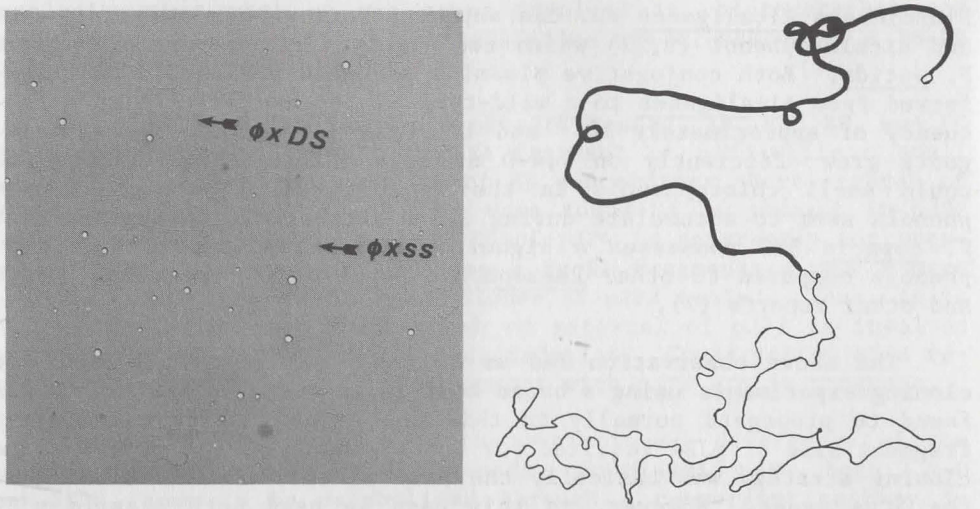

Fig. 7. Electron micrograph of "snap back" structure of the whole molecule of pYG3 showing 25-kb inverted duplication. Diagrammatic representation of the molecule is also shown.

The significance of this sequence rearrangement in the activation of 3Cba pathway is not known. Cells harboring pYG3 plasmid grow efficiently on 3Cba. It will be interesting to see whether the pYG3 form becomes sole plasmid population after prolonged cultivation of Alcaligenes on 3Cba. Such genetic rearrangements leading to $3Cba^+$ phenotype appear to make the cells unable to utilize 2,4-D. The precise map of deletion fusion points and duplicated segments is essential to have an understanding of the role of this unique structural rearrangement in functional expression of 3Cba genes.

Structural and Functional Interrelationship Among the Plasmids Isolated from Chlorophenoxyacetate-Degrading Bacteria

Cloning of the 2,4-D degradative genes. In the case of 2,4-D degradation, although all the enzymes for 2,4-D degradation are believed to be plasmid coded, apparent failure to get the expression of this phenotype in P. putida beyond a reasonable doubt, and failure to localize the genes for the first enzyme(s) of the pathway on a plasmid (14) do not exclude the serious question of any essential role of host determinants in 2,4-D catabolism. In our hand, though poor growth has been observed on the 2,4-D plate (600 µg/ml) of some P. putida strains harboring plasmid pJP4, after weeks of incubation (with $pJP4^+$, PpG1900, or PRS2015) all efforts to grow them in liquid culture have failed (I-S. You, unpub. observations). The adaptive status of the host (lack of tolerance to toxic intermediates such as chlorophenols generated during a 2,4-D catabolism) might be a major determinant in such inability of P. putida cells to utilize 2,4-D.

Plasmidless Alcaligenes sp. can support their growth on both phenol and dichlorophenol (9,22) which are highly toxic to organisms like P. putida. Both conjugative plasmids pJP4 and pJP2 could be transferred from Alcaligenes to a wild-type P. cepacia 383 (7) at a frequency of approximately 10^{-6} and 10^{-5}, respectively. The exconjugants grew efficiently on 2,4-D as sole carbon source though one could smell chlorophenols in the vapor phase. Although chlorophenols seem to accumulate during 2,4-D metabolism, it appears that P. cepacia 383 possesses a higher level of resistance to chlorophenols compared to other Pseudomonas as shown by the exconjugants and other reports (7).

The above observation led us to use P. cepacia as a host for cloning experiments using a broad host-range vector pLAFR1 which was found to propagate normally in this host, to identify the minimum fragment size of pJP4 required for conferring $2,4\text{-D}^+$ phenotype. The cloning strategy was basically the same as described in cloning of the 3Cba genes. However, in this case we used both plasmid pJP4 and pJP2 to clone the 2,4-D genes.

Although we did not get any $2,4\text{-D}^+$ clone from the EcoR1 library of pJP4, from the pJP2 EcoR1 library we succeeded in cloning a 20-kb segment containing 2 EcoR1 fragments (EA and EF of pJP2). This clone was designated as Y21. Further analysis of this clone and detailed biochemical characterization will be reported elsewhere (I.-S. You, in prep.). Recently, several $2,4\text{-D}^+$ clones have also been obtained from a HindIII gene bank of pJP4. Their characterization is in progress.

Structural and functional interrelationships between plasmids pJP4 and pJP2. Plasmid pJP2 is about 20 kb smaller than pJP4 (25). Phenotypically, A. eutrophus harboring pJP2 can degrade both 2,4-D and its nonhalogenated analogue phenoxyacetate but not 3Cba, whereas, isogenic strains of Alcaligenes harboring pJP4 can utilize 2,4-D, 3Cba, and 4Cpa but not phenoxyacetate (14,22,25).

Our success in cloning 2,4-D catabolic genes in a 20-kb segment from pJP2 and initial failure to get such clones from pJP4 led us to speculate that 2,4-D genes including regulatory sequences may be present in a more clustered fashion on pJP2 compared to pJP4.

Supporting evidence in favor of this hypothesis has been obtained from a series of hybridization experiments in which individual EcoR1 fragments of pJP4 (EA, EB, EC/ED, EE, EF, EG, and EH) were purified and nick-translated. Each of this probe was hybridized to EcoR1 digested or SmaI digested pJP2 restriction fragments blotted to nitrocellulose filters. In complementary experiments, EcoR1 fragments EA, EF, and EG of pJP2 were purified and hybridized to various restriction fragments of pJP4. Such experiments in combination with cloning data stengthened the

conclusion that most of the genes involved in chlorocatechol and substituted phenoxyacetic acid degradation are clustered in a 16-kb EcoR1-A (EA) fragment of pJP2.

Among the 8 EcoR1 fragments of pJP4 tested, EB, EE, EF, and EG showed strong homology with the EA fragment of pJP2 while a small segment of EA near the vicinity of EB and another short stretch of EC in the vicinity of EE of pJP4 were found to be present in pJP2 EcoR1-F fragment (EF). Fragment ED of pJP4 is apparently not present in pJP2. Interestingly, these 2 EcoR1 fragments A and F were recovered from 2 of the 3 2,4-D clones of pJP2 tested. Thus, it is logical to assume that about 40 kb of material of pJP2 is involved in other functions besides 2,4-D degradation. Construction of a restriction map of pY21 and its alignment with pJP2 are in progress.

It is interesting to note the evolutionary divergence of the functional aspects of these 2 plasmids. In plasmid pJP4, both 2,4-D and 3Cba, seem to be catabolized through a convergent pathway in which monochlorocatechol is a common intermediate, as discussed by Weightman et al., (14) and others (25). In support of the above view, 4Cpa was found to serve as a growth substrate for Alcaligenes sp. harboring pJP4 (14). However, the degradation of 2,4-D through a dichlorocatechol intermediate is a rather common pathway in nature (31), in which the removal of chlorine occurs after ring cleavage.

Since A. eutrophus harboring pJP2 is 3Cba^-, it is likely that 2,4-D degradation pathway encoded by plasmid pJP2 proceeds via 3,5-dichlorocatechol. It was repeatedly observed that A. eutrophus harboring pJP2 grows faster in 2,4-D medium than that containing pJP4. This phenomenon could be explained by the fact that dehalogenation by lactonization, followed by HCl elimination, is energetically the most favored mechanism (31). In summary, the divergence of 2,4-D pathways and the convergence of 2,4-D and 3Cba pathways may occur at dichlorophenol and chlorocatechol stages respectively.

Structural and Functional Interrelationships Between 2,4-D Plasmid pJP4 and Plasmids Isolated from 2,4,5-T-Degrading Strain of P. Cepacia

Unlike 3Cba and 2,4-D degradation, proof for the functional involvement of plasmids in 2,4,5-T degradation is lacking. Recent evidence from the work done in this laboratory, however, indicates a probable role of resident plasmids of P. cepacia strain AC1100 in 2,4,5-T degradation.

Total plasmid DNA has been isolated from this strain and physical characterization has revealed some heterogeneity. The most predominant population (80% or more) consists of a single size plasmid of 170 kb ± 5 kb. Approximately 10% of the population had a

size of 40 kb. These 2 plasmids were designated pDG3 and pDG4. The rest of the molecules were of sizes varying from 2 kb to 30 kb and were least predominant as judged from electron microscopic analysis. Curing of strain AC1100 of the resident plasmids using conventional techniques of mitomycin C treatment has met with failure. The instability of the 2,4,5-T phenotype especially in absence of selection pressure is a major problem in isolating plasmid-free 2,4,5-T derivatives.

In our preliminary study, we introduced RP4 or R68.45 into a strain derived from AC1100 (DC102, 2,4,5-T$^+$ Nal^r) and repeatedly subcultured under strong antibiotic selection. From such populations, we isolated derivatives of strain AC1100 that appear to have lost some resident plasmids as judged from the restriction analysis of plasmid DNAs isolated from 2,4,5-T$^-$, Kan^r, Tet^r, and Amp^r mutants. RP4 harboring DC102, 2,4,5-T$^-$ derivatives lacking plasmid DNA can grow well on salicylate indicating that salicylate-degrading genetic markers are present on the chromosome. A wild-type P. cepacia 383 (7) was also found to be Sal$^+$ and did not harbor any detectable plasmid. No homology was detected between a known SAL plasmid and AC1100 plasmids in Southern blotting experiment.

Since 2,4-D and 2,4,5-T are structurally analogous, it seems reasonable to assume that the genes encoding 2,4,5-T related catabolic functions may have some homology with 2,4-D degradative genes as part of their common evolutionary ancestry. In agreement with this assumption, a region of homology has been detected ($\sim$ 4 kb) between pAC1100 (a composite of AC1100 plasmids) and a segment of the EcoR1 fragment (EB) of pJP4 which harbors the structural genes for both the 2,4-D and halocatechol degradation. Additional regions of homology between these 2 plasmids have been detected, especially in the region of putative regulatory functions for halocatechol catabolism on the pJP4 physical map. Because of the large size of pAC1100, lack of restriction map, and other recognizable physical and genetic markers, analysis of the precise functional role of the plasmids in 2,4,5-T degradation has not yet been possible. Furthermore, plasmids from AC1100 are isolated in poor yields from batch cultures grown on 2,4,5-T, presumably because of a low copy number of these plasmids. Presence of extracellular DNAase in P. cepacia may also greatly reduce the yield of the plasmid DNA.

In spite of the significant homology detected between pJP4 and resident plasmids of AC1100, the fact that 2 catabolic pathways seem to have undergone drastic evolutionary divergence was suggested from an induction experiment. Induction of 2,4,5-T catabolic pathway with 200 µg/ml of 2,4,5-T did not support any detectable growth of P. cepacia DC102 in minimal medium containing 800 µg/ml 2,4-D. 2,4,5-T grown AC1100 cells are known to completely dechlorinate 2,4-D, but are unable to grow with this compound since the dechlorinating enzymes are not induced by 2,4-D.

Both pJP4 and pJP2 could be transferred at low frequency (10^{-8} and 10^{-7}, respectively) to the strain DC102 resulting in new hybrid strains, designated as IY43 (2,4-D$^+$, 2,4,5-T$^+$, Hg^r, Nal^r) and IY25 (2,4-D$^+$, 2,4,5-T$^+$, Nal^r), respectively, capable of growing with both 2,4-D and 2,4,5-T. In strain IY25, both plasmid pJP2 and pAC1100 exist in unmodified form whereas exconjugants containing pJP4 (6, including IY43, tested) showed a deletion of a 10-kb fragment of pJP4 but pAC1100 appeared to be unmodified (I.-S. You, in prep.).

Because of the significant homology of the 4-kb segment between the plasmids, pJP4 and pAC1100, it was anticipated that recombinational events might generate hybrid plasmids in vivo. Mobilization of these 2 plasmids in different genetic background may help in physical and functional analysis of P. cepacia plasmids. Experiments along this line are presently underway in our laboratory.

CONCLUDING REMARKS

The extensive homology observed between pAC27 and pJP4 concerning the chlorocatechol-degradative genes and that between the same regions of pJP4 and the resident plasmids of the 2,4,5-T-degrading strain of P. cepacia AC1100 is not only interesting, but it also points to the role of plasmid genes in the evolution and spread of degradative characters against toxic chemical compounds. Although this phenomenon is well-known in the evolution and dissemination of antibiotic-resistance genes, no clear evidence for the occurrence of a similar trait in soil bacteria was available until now. The structural studies presented in this paper also point to the important role of direct and inverted repeats in the genetic rearrangements necessary for rapid transition from growth on one substrate to that of a different substrate. The amplification of the structural genes in absence of a putative positive regulatory element is analogous to that observed in case of antibiotic resistance to allow the host microorganisms to withstand high and toxic concentrations of such antibiotics (32,33). Many toxic chemicals resemble antibiotics in terms of their inherent toxicity, both toward animals as well as microorganisms, and it is little wonder that microorganisms employ essentially identical tools, i.e., deletion, fusion, insertion, and other modes of genetic rearrangements, to help evolve new degradative functions. Continued studies of such processes are expected to provide unique insights regarding the mode of evolution of new biological functions in bacteria.

ACKNOWLEDGEMENTS

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NOTES ON METABOLIC PLASMID ORGANIZATION AND EXPRESSION

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METABOLIC VERSATILITY AND PLASMIDS

The term metabolic plasmid, as opposed to resistance plasmid, refers to the encoding of multistep reaction sequences leading from noncellular structures to anaplerotic intermediates. The alternate term, degradative or catabolic plasmid, also denotes the control of these predominately energy coupled processes yielding principally the 2 to 4 carbon acids whose metabolism is chromosome encoded. The aromatic and terpene oxidative pathways, for example, each comprise more than a dozen reactions, several catalyzed by multiprotein enzyme systems. The genes are organized in 2 or more clusters under positive pleiotropic and secondary control. Figure 1 illustrates the phenotypes encoded by the "terpene" plasmid CAM, the "aromatic" ones NAH and SAL, and the "heterocyclic" NIC plasmid. For illustration we shall consider the state, structure, and control of CAM and of NAH/SAL. A related aromatic plasmid, TOL, will be considered by participants who have made the primary contributions.

Many, perhaps most, metabolic plasmids are self-fertile and range in size from ca. 100 to $\geq$ 200 kilobases (kb). They are scored on minimal media as positive-growth phenotypes. In contrast, the smaller resistance plasmids are scored on complete media and usually carry one, or a few, genes that encode enzymes to modify the procaryote toxic agents. The fertility regions of about 40-50 kb contribute at least in part, along with the multigene, multi-regulatory units, to the large sizes.

Metabolic plasmids were first recognized during our search for the genetic basis for the conversion in nature of organic structures to biosynthetic intermediates and to atmospheric CO_2. The first hint of an extra-chromosomal gene array came from lack of transductional linkages between the control of terpene oxygenation and auxotrophic loci in fluorescent pseudomonads, known to be prime partici-

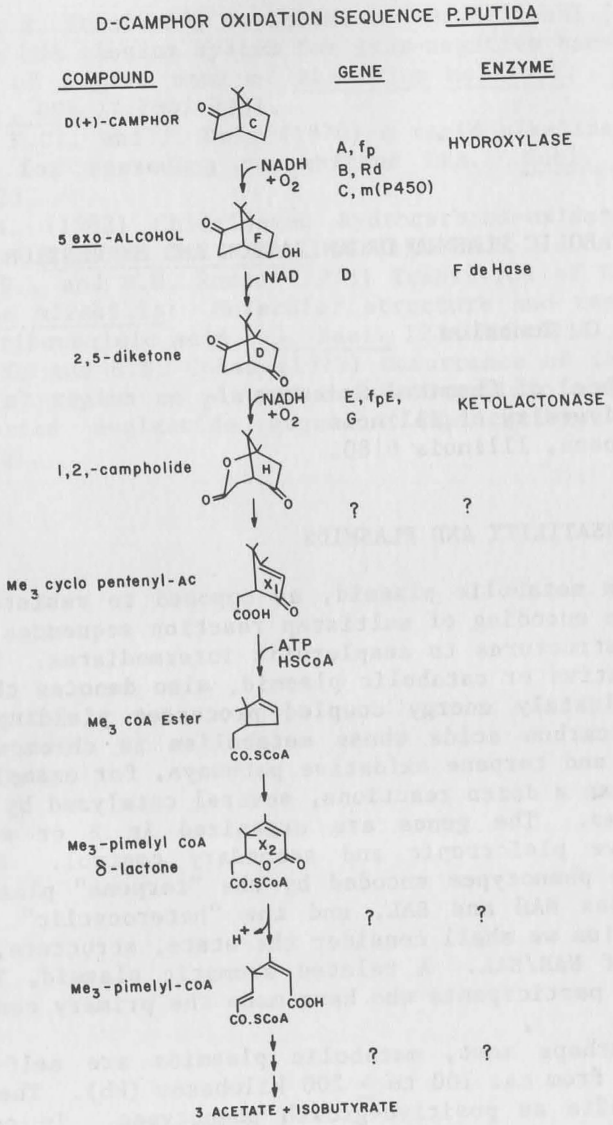

Fig. 1. Examples of phenotypes encoded by metabolic plasmids.

A. CAM, D-camphor to $3C_2 + C_4$ products.

pants in cycling carbon in the biosphere (1). The second signal came from the spontaneous, then induced, loss of entire pathways-so-called "curing" or Seg mutants. As the chemical intermediates became available for genotypic scoring, linkages were soon found for sequential steps along the metabolic pathways. Plasmid fertility was established, an additional role in chromosome mobilization docu-

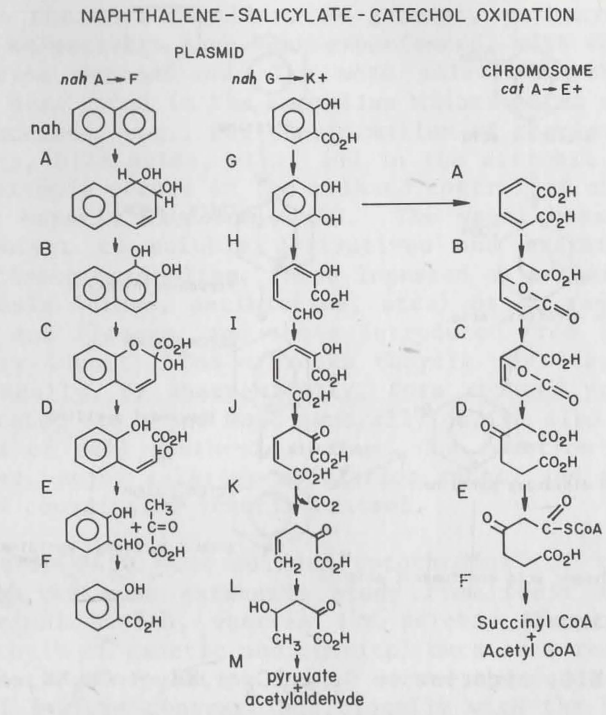

Fig. 1. (Continued)

B. NAH, naphthalene/salicylate to $C_1 + C_2 + C_3$, or to $C_2 + C_4$.

mented, and plasmid chromosome gene redundancy found at the isobutyrate level as we observed co-induction with the metabolism of the branched chain amino acid valine as a carbon source.

To inquire of a general, versatile role of plasmids, we selected the aromatic structures whose metabolic intermediates and pathways had been established, mostly by the Dagley and Evans schools (2). Noel Dunn arrived from the Holloway laboratory, examined the genetic base of naphthalene oxidation and validated a NAH plasmid, pDG7, in a stock culture strain, ATCC 17485. Two phenotypes, Nah^+ and Sal^+, were established and the plasmid implicated in the catechol meta cleavage. The ortho fission pathway was shown to be chromosome-encoded, and under inductive control at the level of catechol an early product thereof (3). Subsequently, general occurrence of plasmids carrying the genes for aromatic oxidative processes was recognized. The naphthalene-positive strains from the Stanier et al. collection (4), whether curable with mitomycin or not, have been shown by Patty Harder to carry plasmids with restrictase patterns compatible with pDG7. Today, TOL (5) is the best

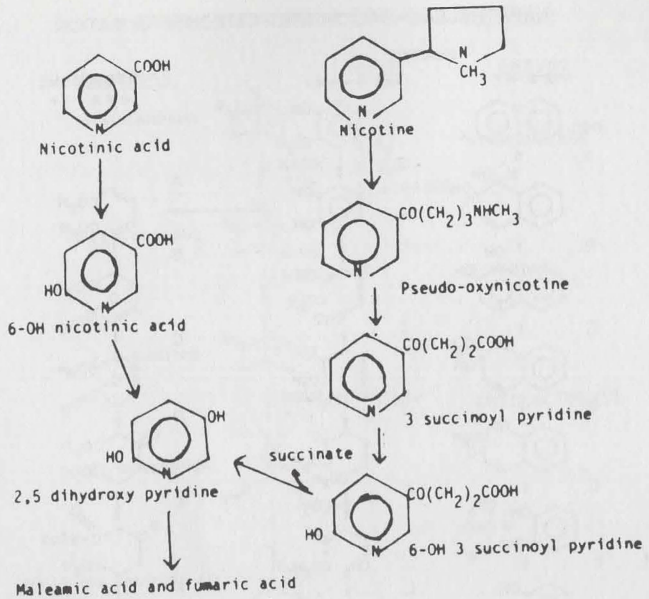

Fig. 1. (Continued)

C. NIC, nicotine to $C_1 + 2C_4 + NH_3 + CH_3NH_2$.

documented plasmid. In host strain mt-2 it specifies the genetic organization of the toluene-xylene oxidative patterns. The relevant data appear in this volume from the 3 principle groups, Williams in Bangor (5), Timmis (6), and Bagdasarian (7) in Berlin--now Geneva and Umea, respectively--and the Nakazawa laboratory (8) at the University of Yamaguchi in Japan.

The abundance and variety of organic structures released into the biosphere challenge the natural carbon cycling processes of nature in breadth of genetic and catalytic questions, many of whose solutions remain obscure (9).

Genotype Scoring

The mammalian liver and kidney microsomes and the microbes of the soil and water serve similar, prominent roles in the removal of these structures. Both have been shown to a major extent to activate molecular dioxygen at the expense of redox energy with direct incorporation of [0] or [0-0] into substrate at otherwise inert loci. The primary oxidative process occurs at a heme iron atom bearing a cysteinyl thiolate axial ligand which can serve as a 2 electron reservoir prior to H_2O release. Examples of plasmid versatility and the relevance of the catalytic centers can be illustrated by their role in encoding the oxygenases that induct hydrocarbon

structures to the more familiar, or general, pathways. Two solutions to the selectivity have been experienced, with control in both gene and enzyme expression. The more selective, stereospecific, systems have been found in the mammalian mitochondria among the biosynthetic processes, e.g., for the formation of regulatory steroids, prostaglandins, bile acids, etc., and in the microbic processes. A contrasting example occurs in the relaxed control of steric requirements in the hepatic microsomes (9). The usual hepatic and renal processes convert to soluble derivatives and excrete the normal products of tissue metabolism, those ingested as pallative or therapeutic chemicals (drugs, antibiotics, etc.) or as residues of food constituents and flavors, and those introduced from the atmosphere as respiratory insult. The microbes recycle most carbon compounds to CO_2 aerobically, or anaerobically, form reduced products, e.g., methane, acetate, etc., and most generally derive also therefrom the intermediates of cell synthesis. Thus, the reaction sequences are more extensive, enjoy a larger modulation range, and are under more clustered and coordinated genetic control.

The hepatic P450 heme sulfur "cytochromes" of the microsomes have received the most extensive study from their implication in human and animal health, whereas the aerobic microorganisms have provided the bulk of genetic and chemical data as a result of amenability to genetic manipulation and of enzyme abundance and activity. The microbial systems contrast additionally with the hepatic microsomal ones. The hepatic enzymes were found to be of low affinity for a broad range of substrates with relatively low turnover, whereas the microbial enzyme systems are more selective with higher substrate affinities and turnover.

The gene-enzyme components and reactions of 2 microbial systems for the terpenes are illustrated in Fig. 2. The electron flow does not pass to the heme in the absence of substrate and thus does not dissipate cellular energy. Three genes, camABC, under coordinate regulation specify the 2 redox effector auxiliary proteins, and the substrate-selective monoxygenase $P450_{CAM}$. The 45 kd flavoprotein and a 12 kd iron sulfide protein are encoded, respectively, by camA and B; the camC product, a 45 kd $P450_{CAM}$ by camC, the following camD locus product is, an alcohol dehydrogenase composed of 2 equal size ~40 kd subunits.

GENETIC DISTRIBUTION

The information among procaryote strains is carried in the population. Cells selected by enrichment usually express but part of the total metabolic potential. The general distribution of traits, the homology among genomes and related pathways follow from the broad host-range plasmid fertility and their ability to initiate chromosome transfer. Relatedness is also indicated by the plasmid incompatibility groups, for example, CAM and OCT in P2 and NAH and

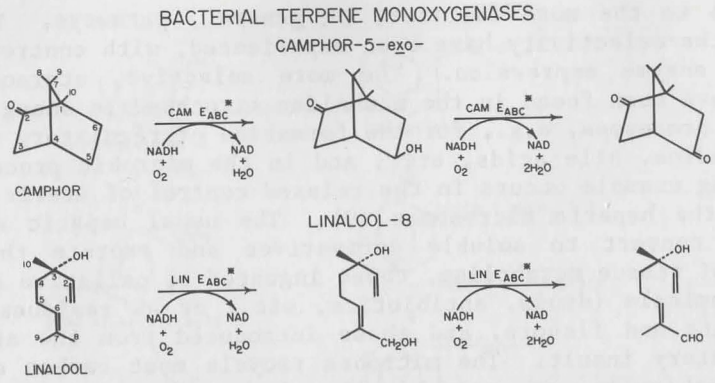

Fig. 2. Terpene P450 hydroxylase components - CAM/LIN.

SAL, P9 (28). In our hands convenient metabolic plasmid examples have included those that control terpene and aromatic oxidative pathways (5,28). More recent evidence suggests other terpene pathways under plasmid control, namely linalool, LIN. Preliminary reports have characterized a plasmid encoding heterocyclic metabolism of nicotine, NIC and nicotinate (29).

Although the CAM plasmid emerged first from our explorations, the aromatic plasmids, which followed, have provided the better method development and permitted more rapid progress in the analysis of structure and regulation (10-14). They are thus preferable examples.

Naphthalene/Salicylate

The naphthalene-positive phenotypes may utilize also salicylate, an intermediate of the first ring fission and pyruvate release, as shown in Fig. 1B. Thus, Nah$^+$ is a growth phenotype with cell yield equivalent to pyruvate, and Sal$^+$, a second one that provides through meta fission pyruvate and acetaldehyde or by the ortho pathway the succinyl- and acetyl-CoA derivatives. Aromatic structures with aliphatic substituents may yield ketones rather than aldehyde. These generalities apply to most aerobic, saprophytic cells of soil and water; of these we have used the fluorescent pseudomonads as biological prototypes (1,12,15).

A *Pseudomonas putida* strain PpG7 bearing the plasmid pDG7 expresses the inducible phenotypes Nah$^+$, Sal$^+$, with ring fission via the meta pathway (3). Chakrabarty (16) reported a plasmid, pAC1, of phenotype Nah$^-$Sal$^+$. The 2 are homologous except for an altered *nahA* gene locus (11-13), Fig. 3B. Among recent isolants, and culture collection strains, including several from the Rothamstead and Evans

laboratories (4), were observed to carry one or more plasmids resembling pDG7 and pAC1.

Clues to the evolution of convergent and alternate genetic patterns among plasmids have been provided by the numbers of strains, the array of chemical intermediates, reactions and pathways observed with substrates from fossil fuels and products of current plant, animal, and synthetic chemistry. Among the convergent trends one observes the fission of catechol, and analogs, and the homology among genes for the meta pathway irrespective of the initial substrate, e.g., naphthalene/salicylate vs. xylene/toluene. Degeneracy may appear in the gene regulation with some retention in the enzyme complement. The range of structural tolerance among substrates is accompanied by mutation, altered gene array and regulation. For example, Yen's selection of Sal$^+$ revertants from Sal$^-$ strains were found to delete plasmid genes which resulted in action of chromosomally encoded pathway, i.e., the ortho fission of catechol (24).

Alkane and Isoprenoid Gene Analyses

The plasmid-encoded hydrocarbon oxygenation processes of normal, branched, and cyclic carbon structures arose as we extended to genetic analyses the chemical inquiry of the terpene, camphor, and alkane metabolism (30-32). The 10 carbons of the terpene 2-bornanone are returned to CO_2 via 3 acetate and the debranching of isobutyrate to succinate. The bicyclic ones with ketone oxygen function gain solubility with less toxicity and hydrophobic rejection from the enzyme centers than the alcohols. Add the interesting stereochemical riddle presented by the examples of a hydroxylation of both the D- and L- enantomer of camphor by the single $P450_{CAM}$ enzyme center, indicated in Fig. 2, and a wedge to stereochemical permissiveness and group affinity is presented for refinement in a stringent enzyme model.

The acyclic monoterpenes are also prevalent plant products. Linalool, for example, with an assymetric (chiral) essential oxygen center at carbon 3 and 2 olefin centers exhibits configuration and binding requirements. Linalool-competent strains produce, under induction, a $P450_{LIN}$ system with stringent substrate requirements in a fluorescent pseudomonad. This curable phenotype presents an added access to genetic fine structure, some 60-odd analogs of linalool have been prepared to explore the heme-substrate site. From the exploration of chemical variety, features of homology emerge. For example, the system parallels of the camphor-5-*exo*-methylene and linalool-8 hydroxylases are indicated in Fig. 2. The enzyme systems differ only in the detailed primary and tertiary structure around the active center and in component interaction domains. The $P450_{LIN}$ active center probes extended to analogs modified at each of the 10 carbons and the essential oxygen function define first-order effects of spatial and affinity properties of the heme pocket (19).

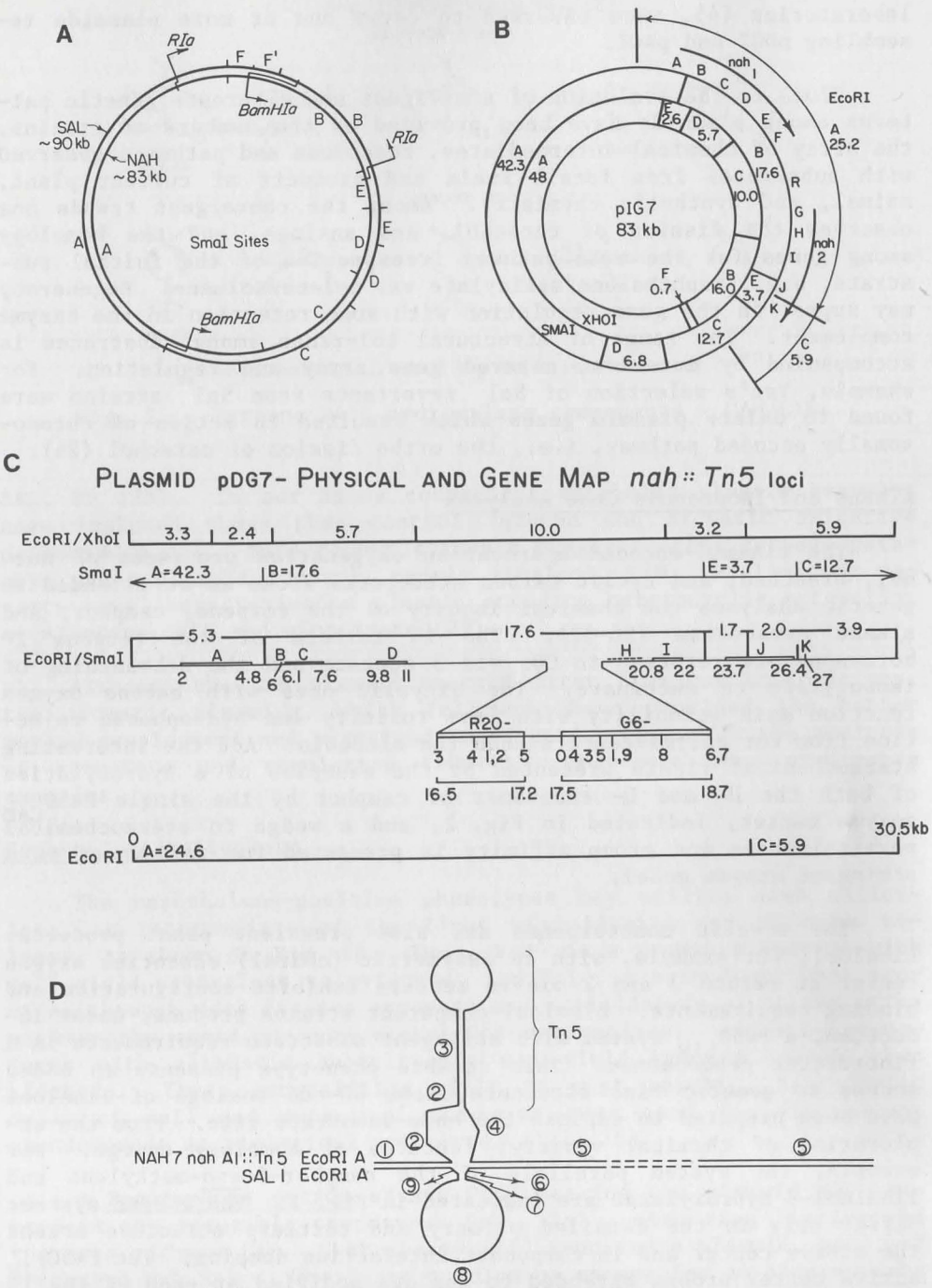

Fig. 3. Aromatic metabolic plasmid DNA: NAH(pDG7) + SAL(pAC1).
A. Circular SmaI restrictase maps.

Fig. 3. (Continued).

B. Structural and regulatory gene clusters of pDG7.

C. Nah gene organization: three operons, nahAF, GK, and nahR.

D. Heteroduplex of insertion/deletions, in nahA locus of pAC1/pDG7.

A third large class of terpenoid structures of the monocyclic group has been probed. Through para-cymene a bridge is offered to saturated and aromatized structures from a p-methyl and p-isopropyl base. Bhattacharyya et al. have developed the p-cymene system (20) and to a lesser extent the P. aeruginosa strains reported to metabolize geraniol (4); the redox and aliphatic vs. aromatic variety are added by the P450 para-cymene components. A $P450_{PCM}$ has been p-isolated; it responds to the inducing substrate p-cymene and numerous related saturated structures. The active center does not accommodate camphor nor linalool. The enzyme components in heterologous recombination exhibit affinities and limited catalysis.

The structure-function parameters of $P450_{CAM}$ have provided a prime model to physical, chemical, and genetic probes. The microbial origin affords control, quality, and abundance (15,21). The advances to the oxygenase field include primary structures of 2 components, the third well advanced. The carbon and O_2 center of $P450_{CAM}$ provides 2 regions with Cys residues with amino acid homology to other P450 systems including the hepatic microsomal enzymes (22,23). The current, working model targets 1 of the Cys residues as the essential sulfhydryl axial ligand to the heme (21-23) to provide the P450 character of the heme sulfur proteins. The unique 2 electron redox center is attributed to a retained sequence (26). A tertiary structure of $P450_{CAM}$ is now nearing completion with much anticipation of fine structure disclosure of binding, selection, and motion in catalysis (27).

HOST RANGE OF A PLASMID DNA EXPRESSION

As the methods for small plasmid DNA isolation were developed particularly in E. coli, we attempted their application to the larger metabolic plasmids of genus Pseudomonas. Early variations met with limited success prior to James Johnston's joining our laboratory with the primary desire to solve the DNA isolation problem and to document the genetic components by Pseudomonas transformation. Sporatic successes in a learning period blended into a predictable routine to yield circular plasmid DNA from metabolic plasmids of less than or equal to 100 kb. The primary focus on the NAH pDG7 plasmid was soon extended to the related TOL and SAL molecules

with restrictase characterization and visualization by electrophoresis (10).

With the help of Jean Ito, Irving Crawford and I (11) found, by the first restriction analysis of NAH and SAL DNA preparations furnished by Johnston, similar fragments with the exception of a single larger component from SAL. SmaI was soon found to provide 5 near-identical size of fragments, and SAL 2 additional ca. 3 kb fragments. In EcoRI digests the latter arose in SmaI redigests of the EcoRI fragment A (11,12). Subsequently, Farrell modified the methods and carried out a broad spectrum of restrictase characterizations. These demonstrated NAH and SAL equivalence in Southern heteroduplex blots. The 117 kb TOL DNA anneals at about 70% homology with the 80 kb NAH. These data followed by transformation, with methods but slightly modified from the E. coli procedures of Helinski, confirmed and extended the earlier analyses by transduction and conjugation. The interstrain transfer, via transduction or conjugation, of the complete Nah^+ and Sal^+ phenotypes to the PpG1 cell line in strain 277 (trpB615ΔCAM) provided the a basis fof the genetic analysis of the organization and regulation of the plasmid-encoded aromatic metabolism.

The very large size of the terpene-encoded plasmids, for example, the CAM plasmid, has not yielded to routine isolation. Thus, the breakthrough of Koga to clone from a CAM plasmid DNA library with expression in Pseudomonas and E. coli has opened a route to full genetic participation. In EcoRI the pKT240 vector is amply expressed presumably from the Ap^r promoter; in vector pBR322 similar expression ensues from the same promoter. The camC gene product $P450_{CAM}$ 412 of amino acid residues is formed constitutively from a 2.2 kb insert; the genetic organization of these systems on the plasmids will be discussed. The Pseudomonas hosts express the camC insert of vector pKT240 constitutently from an unidentified promoter at levels equivalent to about twice the expression in E. coli from the vector pBR322.

PLASMID ORGANIZATION

Aromatic/Naphthalene

The circular restriction maps of the DNA from the NAH, SAL, and TOL plasmids, respectively pDG7, pAC1, and pWW0, were prepared. The intact DNA was shown to transform plasmid cured strains of Pseudomonas putida from both the homologous and a variety of heterologous cell lines (6,16). The pDG7 plasmid, first analyzed by Dunn, was subjected to Tn5 insertion mutagenesis by Yen (12). The insertion loci were determined by restrictase digestion and the genotypes scored by enzyme assay to define gene order and locus as shown in Fig. 3B. Two gene clusters were found, each of about 10 kb, bracketing a regulatory region of about 1 kb. These are indicated in more detail in Fig. 3C. The 2 gene clusters, under pleiotropic control by salicylate indicates 2 operons mediated by the locus nahR. The nahR locus maps just prior to the nahG locus of the second operon about 5 kb downstream from nahD. The nahEF loci specifying an aldolase and aldehyde dehydrogenase could reside in this region. The more detailed map, prepared by scoring the gene products of some 50 Tn5 inserts suggests the gene boundaries. They may be extended as additional mutant loci are identified. Especially to be noted is the rather extended region assigned to genes nahA and J. Gibson et al. have defined 3 protein components of the naphthalene hydroxylase; thus 3 genesare encoded within the 3 kb informally denoted as "nahA."

Homology of the NAH and SAL plasmids is apparent as indicated by the EcoRI and SmaI restrictase fragments, Fig. 3A, and by heteroduplex microscopy annealing and electron measurement. The EcoRI A fragment from pDG7 mutant nahA1::Tn5 vs. the wild-type pAC1 as shown in Fig. 3D with the Tn5 inverted repeat mapped as marker at 2.6 kb from the left margin of the EcoR1 fragment A, the SAL plasmid pAC1 shows a short inverted repeat at about 100 kb, and the longer insert, indicated by 8 which has not annealed with the pDG7 nahA gene locus. The Tn5 insert also makes visible small segments of nonannealed nahA as indicated by 2, 2', and 4 in the figure. The nature of the SAL insert and deletion will require added DNA analysis. These lesions and the pleiotropic control result in a full Nah^+ operon.

In summary, the Nah^+ and Sal^+ phenotypes of pDG7 result from 2 operons under pleiotropic control of salicylate or anthranilate (2-aminobenzoate). They account for about 30 kb of the 80 kb plasmid. Should the fertility functions require 40 kb of DNA, one would anticipate few additional genes in this aromatic plasmid.

VECTORS AND PROMOTERS IN PSEUDOMONAS AND E. COLI

The cell lines which have proved most useful in our analysis of the aromatic and terpene plasmids were derived from the wild-type CAM isolant PpG1. The CAM cured (Δcam) plasmid-free hosts PpG277 (trpB615Δcam) and PpG1343 (met-616Δcam) are preferred stable auxotrophs for transformation and expression. They serve also classical genetics for pf16 transduction, conjugation, and introduction of plasmid fertility. The preferred E. coli strains carry RR1 or are LE392 or HB101.

Broad host-range, multicopy vectors differing in compatibility have been prepared respectively from the vectors RSF1010, IncP4 and pRK2, IncP1. The derivatives used are pKT240, from RSF1010 and pRK290 from pRK2, kindly supplied, respectively, by Timmis and Bagdasarian, and Helinski. Figure 4A indicates their structures and sizes, ∿12 and 20 kb respectively, each suitably expressed in the P.

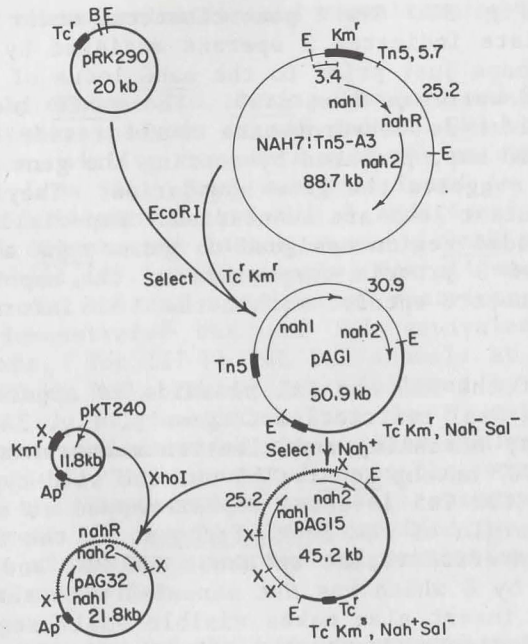

Fig. 4. Aromatic plasmid cloning and expression.

A. pDG7 subclones in compatible vectors pRK290/pKT240.

putida cell line; the vector pBR322 also expresses Pseudomonas genes in E. coli at similar enzyme levels.

The pKT240 vector expresses pseudomonad genes in the Pseudomonas and E. coli hosts, at approximately equal level, and in the latter pBR322, is an equally effective vector. Both pKT240 and pBR322 carry ampicillin, an Ap^r promoter upstream in the PstI insertion site from which expression occurs, whereas in the pseudomonad the pKT240 expression of pseudomonad insertions occurs from a promoter of unknown structure. In our experience, the difficulties encountered by some authors in obtaining expression of pseudomonad genes in E. coli, derives at least in part from attempts to use Pseudomonas promoters rather than from differences in the DNA expression per se (5,7,13,16).

DNA libraries have been prepared routinely from the aromatic plasmid DNAs in the case of NAH, SAL, and TOL. The constructs from the Tn5 insertion mutant, in nahA, were prepared first in pRK290 from an EcoRI library with Tc^r or Km^r selection. The $Nah^+ Sal^-$ clones resulting from the Tn5 insertion were selected for Nah^+ indicating an effective promoter and subclone into pKT240 and an XhoI site by selecting for $Ap^r Km^s$ clones. The inserts were mapped as

shown in Fig. 4B. The subclones and their genotypes are indicated in Tab. 1.

Evidence of Positive Control

The subclones from pAG15 shown in Fig. 4B and Tab. 1 indicate expression by complementation with the nahR region carried in pKT240 in trans array. Complementation with pAG26 and 28 are indicated in Tab. 1 to occur at wild-type level. These data are most readily interpreted as a positive control mechanism.

EXPRESSION CLONES OF THE camC GENE: THE $P450_{CAM}$ MODEL

The CAM plasmid, pRG1 > 200 kb has eluded thus far routine isolation. Occasional success and selection for transcomplementation of the plasmid genes (1) from PstI in the restrictase digest or the use of whole-cell plus plasmid DNA have provided a route to suitable $P450_{CAM}$ and the related expression clones. The selection method is for growth on camphor in strain PpG543 (camC100); selection for Cam or Km^r markers provided a series of Cam^+ strains. Following for selection for rapid growth, plasmid DNA was isolated by the usual procedure (11) and the DNA used to transform strain PpG277 (trpB615Δcam). Clones were grown in PAS-Cam medium with a monosodium glutamate as a secondary substrate and screened for P450 production, supplemented with tryptophan. The ferrous-CO - ferrous spectra provided the expression levels of $P450_{CAM}$ indicated in Fig. 5D.

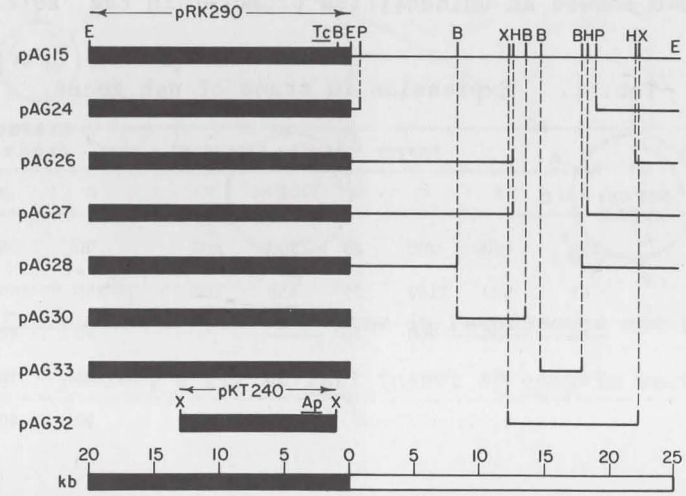

Fig. 4. (Continued).

B. pAG15 subclone expression with nahR in trans.

700

nah OPERON HYBRID AND DELETION PLASMIDS – pRK290-pDG7

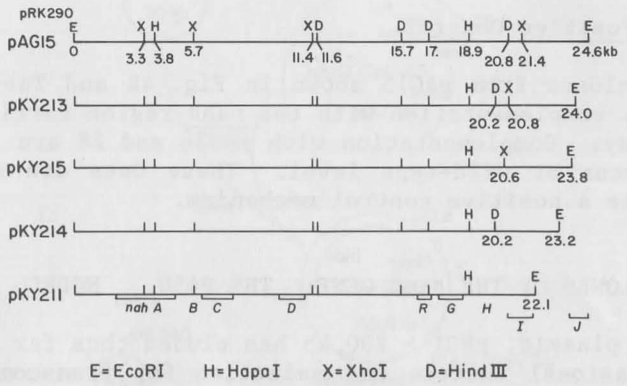

Fig. 4. (Continued)

C. Phenotypic revertants express aromatic ortho fission.

Defective strains were subcloned into the Pst site of pBR322, selected for $Tc^r Ap^s$, then screened for P450 production. As indicated in Fig. 5D, the plasmid pKG300 produced ample P450 and E. coli RR under inducing conditions. As indicated by the data in Fig. 5A, the expression of both vectors in E. coli as from the ampicillin-resistant promoter, upstream to the PstI insertion site, i.e., plasmids pKG202 and 300, whereas in the pseudomonad, expression from vector pKT240 showed an unidentified promoter in the BstEII region

Tab. 1. Expression in trans of nah genes.

Plasmid	Enzyme Activity Expressed - nkat[a]								
	A	B	C	D	E	F	G	H	I
pDG7	100[b]	400	900	20	300	200	90	560	50
pAG15	85	500	1100	25	450	250	150	1000	50
pAG26 + 32	60	300	900	20			40	400	2
pAG28 + 32	65	300	nil				26	190	17
" -- 32					6	20	50	400	5
" 26 --	-	-	-	-			3	4	3
" 28 --	-	-					3	5	4

a) nmoles product/min/mg protein; - = nil; blank = not tested.

b) Induced with 0.3 mM anthrinilate; all noninduced are <.1 to .01 of induced.

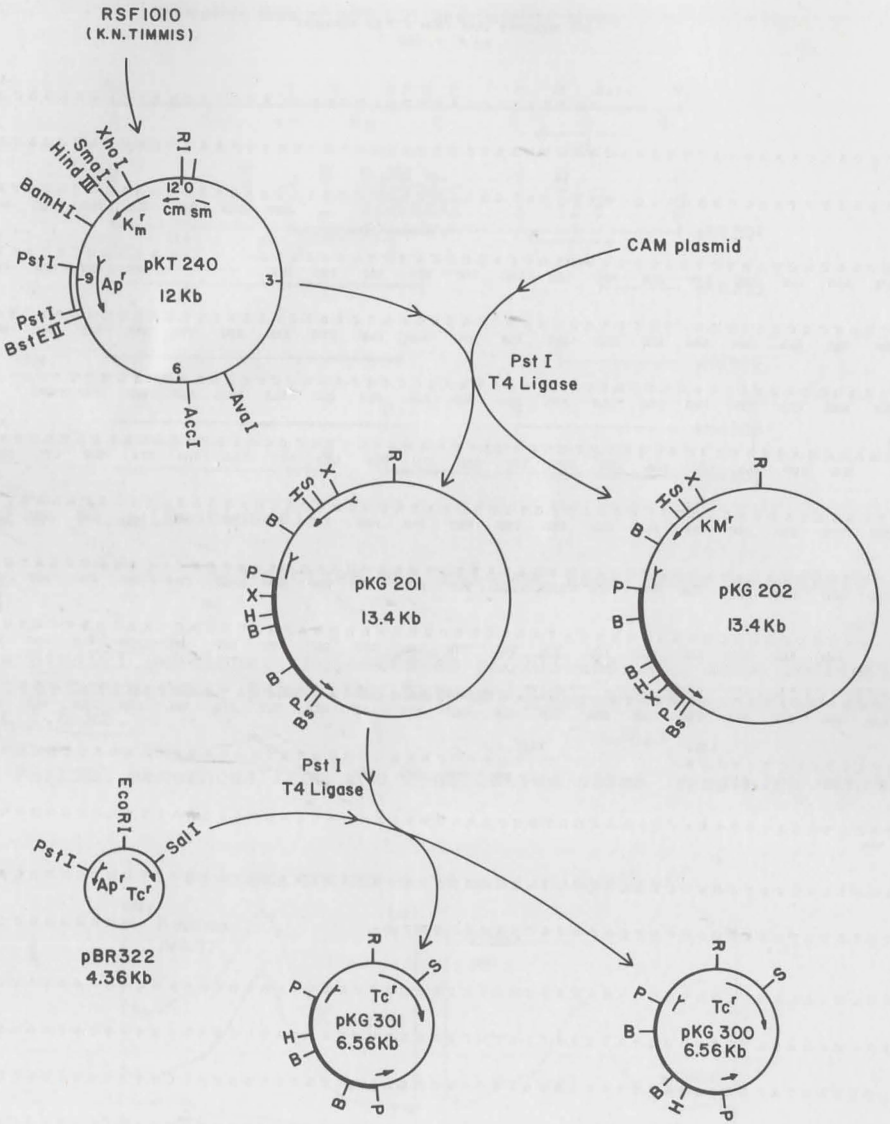

Fig. 5. Expression of the camC gene in Pseudomonas and in E. coli.

A. pKG201, a 2.2 kb PstI insert of camC in vector pKT240.

as indicated in pKG201. These differences are also expressed in Fig. 5D. Figures 5B and 5C concern the 2.2 kb insertion carried in vectors pBR322 and pKT240 whose expression is shown in Fig. 5D together with restriction maps of this region. The 2.2 kb insert expressed in pKG201 was also expressed at the same level in an XhoI

DNA SEQUENCE DATA FROM 2.2 kb FRAGMENT
by S. M. LEE

```
                                                                                                    -100
                                                                                -143
                                                                                  C G C T G G C C G A T C T G A T C A C C C A G C G T T T T T C C A T C G A C G A

                                                                                                    -40
G G C C A G C A A G G C A C T T G A A C T G G T C A A G G C A G G A G C A C T G A T C A A A C C C G C G A T C G A C C C

                                                                                                    1                                                   21
C A C T C T T T A G C C A A C C C G C G T T C C A G G A G A A C A A C A A C A A T G A C G A C T G A A A C C A T A C A A
                                                                                                    MET  THR  THR  GLU  THR  ILE  GLN

                                                                                  61                171                                               182
A G C A A C G C C A A T C T C G C C C C T C T G C C A C C C C A T G T G C C A G A G--      --C A A C G G C G G A C A C
SER  ASN  ALA  ASN  LEU  ALA  PRO  LEU  PRO  PRO  HIS  VAL  PRO  GLU                        ASN  GLY  GLY  HIS

                                                                                                                                                    246
T G G A T C G C C A C T C G C G G C C A A C T G A T C C G T G A G G C C T A T G A G G A T T A C C G C C A C T T T T C C
TRP  ILE  ALA  THR  ARG  GLY  GLN  LEU  ILE  ARG  GLU  ALA  TYR  GLU  ASP  TYR  ARG  HIS  PHE  SER

                                                                                                                                    297
T C C A G C G A G T G C C C G T T C A T C C C T C G T G A A G C C G G C G A A G C C T A C G A C T T C N T T C C C--
SER  SER  GLU  CYS  PRO  PHE  ILE  PRO  ARG  GLU  ALA  GLY  GLU  ALA  TYR  ASP  PHE  ???  PRO

386                                                                                                                                                 444
--G G A T C C A G G A G C T G G C C T G C T C G C T G A T C G A G A G C C T G C G C C C G C A A G G A C A G T G C A A C
      ILE  GLN  GLU  LEU  ALA  CYS  SER  LEU  ILE  GLU  SER  LEU  ARG  PRO  GLN  GLY  GLN  CYS  ASN

                                                                                                                                                    554
T T C A C C G A G G A C T A C G C C G A A C C C T T C C C G A T A C G C A T C T T C A T G C T G C T C G C A G G T C T A
PHE  THR  GLU  ASP  TYR  ALA  GLU  PRO  PHE  PRO  ILE  ARG  ILE  PHE  MET  LEU  LEU  ALA  GLY  LEU

                                                                                                                                                    564
C C G G A A G A A G A T A T C C C G C A C T T G A A A T A C C T A A C G G A C C A G A T G A C C C G T C C G G A T G G C--
PRO  GLU  GLU  ASP  ILE  PRO  HIS  LEU  LYS  TYR  LEU  THR  ASP  GLN  MET  THR  ARG  PRO  ASP  GLY

    1080                                                                                                                                            1137
    --C T G G C C C G C C G G G A A A T C A T C G T C A C C C T C A A G G A A T G G C T G A C C A G G A T T C C T G A C
        LEU  ALA  ARG  ARG  GLU  ILE  ILE  VAL  THR  LEU  LYS  GLU  TRP  LEU  THR  ARG  ILE  PRO  ASP

                                                                                                                                                    1197
T T C T C C A T T G C C C C G G G T G C C C A G A T T C A G C A C A A G A G C G G C A T C G T C A G C C G C G T G C A G
PHE  SER  ILE  ALA  PRO  GLY  ALA  GLN  ILE  GLN  HIS  LYS  SER  GLY  ILE  VAL  SER  GLY  VAL  GLN

                                    1211                    1225                                                                                    1260
G C A C T C C C C T C T G G T--          --A C T A C C A A A G C G G N A T A A A C A C A T G G G A G T G C G T G C
ALA  LEU  PRO  LEU                      THR  THR  LYS  ALA          ***

                                                                                                                                                    1320
T A A G T G A A C G C A A A C G A C A A C G T G G T A A A C G T C G G T A C C G G A C C G G A C T G G C T G G C G T T G
***

                                                                                                                                                    1380
A G G T C G C C T T C G G C C T G C G C G C C A G C G G C T G G G A A G G C A A T A T C C G G T T G G T G G G G G A T G

                                                                                                1413                    1430                        1440
C G A C G G T A A T T C C C C A T C A C T T A C C A C C C T T C C--                               --G G C A A A G C C A C
      ***

                                                                                                                                                    1450
A G C G G A A A G C C T G T A C C T G A G A A C C C C A G A T G C C T A T G C A G C G C A G A A C A C C A A C A C C G G

                                                                                                                                                    1560
A G G C C C A G G T A A C G G C T A T C A A C C G C G C C G A C A G C A A G T A T C C T A T C G G A T C G C C G G G C A

                                                                                                                                                    1620
C T G G A T T A C G A C C G G C T G G T A T T G G C T A C C G G A G G G C G T C C A A G A C C C T A C C G G T G C C A G

                                                                                                                                                    1680
T G G C G C A G T T G G A A A G G C G A A C A A C T T T C G A T A C C C C C C T C G A G T G C A T T C C C C G G C A G C

                                                                                                                                                    1740
T G A T T G C G G A T A A C C G T C T G G T G G T G A T T G G T G G C G G C C A C A T T G G C C T T G A A G T G G C T G

                                                                                                                                                    1800
C C A C C G C C A T C A A G G C G A T A C A T G C A C G T C A C C G T G C T T G A T A C G G C A G C C C G G T C T G G A

                                                                                                1829
G C G G G T T A C C G C C C G C C G G A G G C T A C G A G
```

Standard three letter amino acid code used.
*** = termination sequence

Fig. 5. (Continued)

B. Partial amino acid and DNA sequences of plasmid pKG201.

Restiction Map of pKG 201 and Deletion Maps

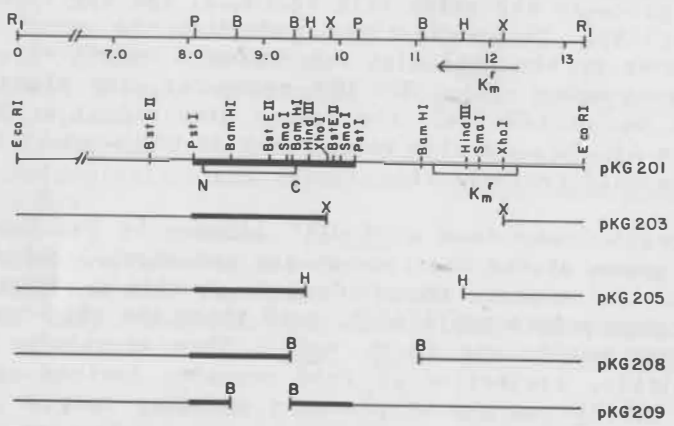

Fig. 5. (Continued)

C. Restrictase map and camC gene locus in pKG201.

and a HindIII subclone, indicated as pKG203 and 205, thus indicating that the structural gene lies between PstI and the HindIII site, about 1.6 kb.

Partial sequences from the restriction sites remaining in the

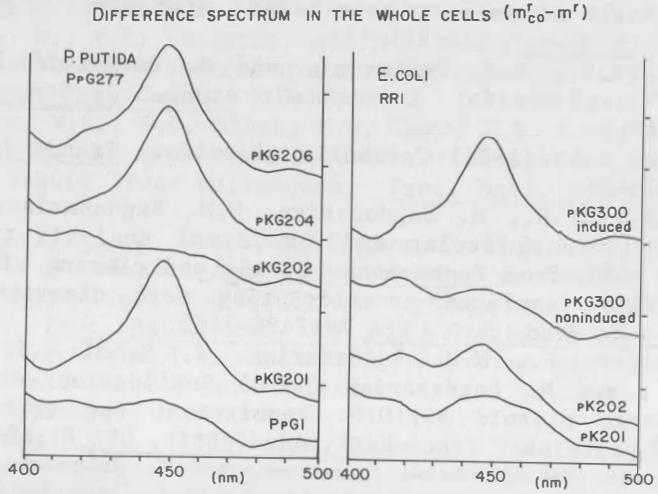

Fig. 5. (Continued)

D. $P450_{CAM}$ spectral assay.

1.6 kb insert are shown in Fig. 5B. The start signal is indicated as nucleotide 1, the known sequence of $P450_{CAM}$ starts with the N-terminal 3, proceeds 412 amino acid residues. At the terminal lies Ala at base 1239. The nucleotides preceding the start signal at about 150 bases to the PstI site are numbered negatively, the last base determined being -143. The DNA sequences stop signals at TAA beginning at nucleotide 1243, the second stop signal at 1261 coincide with the 45,000 molecular weight protein produced by the cloned insert in the wild-type plasmid.

Additional clones from a HindIII library by Dr. Koga express the first 4 genes of the Cam hydroxylase and alcohol dehydrogenase, see Fig. 1A. In summary, these advances provide a vector-promoter system with appropriate expression, 5-10 times the wild-type induced in Pseudomonas putida and in E. coli. They provide a wedge for genetic analysis, projection of P450 protein, further analysis of the control of the metabolic plasmids encoding terpene genes and pathway.

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THE STRUCTURE OF THE mer OPERON

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ABSTRACT

The DNA sequence has been determined for a 3.8-kb region which encodes the mercury-resistance (mer) operon of the IncFII plasmid NR1. The sequence reveals 4 open reading frames which could encode proteins of 12,522, 9,429, 14,965, and 58,912 d corresponding to the 4 previously described Hg-inducible proteins detected in minicells carrying mer^+ plasmids. The Hg(II) reductase protein sequence is about 90% homologous to that of Tn501, but the DNA sequence shows a homology of 60-70% to that of Tn501 except for short regions of very high homology. The entire mer region is 63.4% G-C overall. The region encoding the merR (positive regulatory) function has 3 possible open reading frames, 2 of which overlap in one direction and the third of which reads in the opposite direction. Attempts to visualize the polypeptide(s) encoded by the merR cistron were unsuccessful.

INTRODUCTION

Resistance to mercury compounds is a commonly found plasmid-determined phenotype in both gram-positive and gram-negative bacterial genera (9,30). It occurs in 2 forms. The simpler form of the phenotype ("narrow spectrum") confers resistance only to inorganic mercury compounds, such as, $HgCl_2$ and to a few organomercurials, such as, merbromin and fluorescein mercuric acetate. The more complex phenotype ("broad spectrum") confers resistance to the above compounds as well as to a larger number of organomercurial compounds including phenyl mercuric acetate and merthiolate. There is evidence that these phenotypes are selected in nature by industrial and clinical use of mercury compounds (17,21). Broad-spectrum resis-

tance occurs less frequently than narrow spectrum and both types of resistance have been found on transposable elements in gram-negative bacteria (4,23,29,31). Broad-spectrum resistance involves another enzyme, the organomercurial lyase (13,27), in addition to those functions described below.

Narrow-spectrum resistance to inorganic mercury compounds (mer) is effected by the conversion of toxic ionic Hg [Hg(II)] to the less toxic, relatively inert, volatile elemental Hg [Hg(0)]. This detoxification is effected by the Hg(II) reductase (HR), an enzyme characterized in gram-negative bacteria as a heat-stable, soluble, intracellular, NADPH-dependent, FAD-containing, disulfide oxidoreductase. This inducible enzyme is the product of the merA cistron (10,14,15). Previous biochemical studies have estimated the monomer molecular mass of this enzyme to be from 60-69,000 d. The recently published sequence of the HR encoded by Tn501 indicates a molecular mass of 58,660 (6) for that version of the enzyme. The HR apparently functions as a dimer and shows considerable homology, especially in the active site, to another FAD-containing disulfide oxidoreductase, human erythrocyte glutathione reductase (11,12). The HR is processed proteolytically rather slowly in vivo by the removal of between 3-7,000 d from its amino terminus. Presently, the agent and the functional correlate(s) of this processing are unknown. The HR has also been isolated from a limited number of gram-positive genera and, though less well characterized than the gram-negative enzyme, the gram-positive HR is similar except for heat stability (9,30)

The mer operon also encodes a Hg(II) uptake system, originally designated as merT which is only detectable in mutant strains which have lost the HR and so cannot volatilize Hg(II) (19). Since minicell polypeptide analysis of mer has associated 3 small proteins of approximately 15,000; 14,000; and 13,000 d with this function, the genotype designation merT (10) is inappropriate. However, for simplicity, we retain its use, in quotes ("merT") pending adoption of appropriate designations for the 3 peptides. The larger 2 are inner membrane proteins and the smallest protein is soluble and very rapidly processed to a 12,000-d form in intact minicells (14,15). All 3 proteins are very basic, detectable only by nonequilibrium isoelectric focusing in 2-dimensional polyacrylamide gel analysis.

Genetic studies indicate that both the HR and the Hg(II) uptake system are under positive regulation. As studied on monocopy plasmids, the operon consists of 2 complementation groups: Group A contains the positive regulatory function with apparently only one cistron, merR; and Group B contains both the Hg(II) uptake function ("merT") and the HR (merA). Recent studies of Tn5 mutants of mer in the monocopy plasmid R100-1 indicate that there is a second function (merC), mapping in or very near the amino terminus of the HR which is necessary for expression of the Hg(II) uptake function (3).

Other recent studies (20) with monocopy/multicopy mutant heteroplasmid strains also indicate that regulation may be somewhat more complex than initially posited (16). The positive regulatory function merR appears to be negatively autoregulated (20).

We report here the structure of the operon as revealed by DNA sequence analysis. The DNA sequence itself and a detailed analysis of the polypeptides which it encodes will be presented elsewhere (2).

MATERIALS AND METHODS

Bacterial Strains and Plasmids

DNA for sequencing was obtained from the plasmid pDB7 which carries the mer operon of NR1 cloned directly on a PstI fragment into pBR322. The regulated high-expression vehicle pλ8 (24) maintained in the host strain N4830 was used for subcloning and expression of the merR locus. The recombinant plasmids pRR130 and pRR134 carry, respectively, the entire mer operon and the EcoRI-H fragment of NR1 (18)

DNA Sequencing

The dideoxy chain termination method of Sanger (26) was used as modified by Messing (17). The enzymes Sau3A, RsaI, HaeIII, AluI, NaeI, HinPI, HpaII, and FnuDII were used to generate "shotgun" templates from a 4,000-bp NcoI-StuI fragment of pDB7 which confers the Hg-resistance phenotype when cloned alone into pBR322. Directed clones were also generated using the HincII and EcoRI sites within the mer operon templates. Data were recorded and analyzed using the GENED, GEL, and SEQ programs from Intelligenetics, Inc., Palo Alto, California.

Assessment of HgR Phenotype

Zone of inhibition, efficiency of plating, and Hg(II) reductase assays have been described (3).

Polypeptide Analysis

Labeling of plasmid-encoded polypeptides with ^{35}S-methionine and their analysis by gel electrophoresis and autoradiography has been described (14,15).

Reagents and Enzymes

The Klenow fragment of DNA polymerase was obtained from Bethesda Research Laboratories. Radioisotopically labeled compounds were obtained from Amersham, Inc. Nucleotides, and DNA sequencing

primer were obtained from P-L Biochemicals. Restriction enzymes were obtained from New England Biolabs, Bethesda Research Laboratories, Promega Biotech, and Boehringer-Mannheim. All enzymes were used according to manufacturer's specifications.

RESULTS

DNA Sequence

The 3.4-kb region containing the mer operon begins at the left 38-bp inverted repeat which brackets one end of Tn21 and extends to the termination codon for the HR structural gene. The HindII site and the EcoRI site provided "landmarks" and were used for construction of directed templates. The sequence upon which the operon structure reported here is based is 96% complete on both strands. The single-strand regions include 3 short segments in the HR-structural gene and 1 segment of 30 bp in the merR region. The sequence of the HR of Tn501 reported last year by N. Brown et al. (6) was useful in determining the position of the HR coding region of NR1. The other reading frames described below were chosen on the basis of their being preceded by a reasonably good Shine-Dalgarno sequence (28), the similarity of their codon utilization patterns to that of the HR, and their correspondence in size, amino acid composition, and hydropathicity (16) to polypeptides previously shown to be specified by the operon (14,15). Since Hg(II) interacts with the sulfhydryl group of cysteine (the HR has 8, Ref. 6), we have also been concerned with the number of cysteines in each predicted protein.

There are 3 possible open reading frames in the left region of the operon (Fig. 1). This is the region previously shown to encode

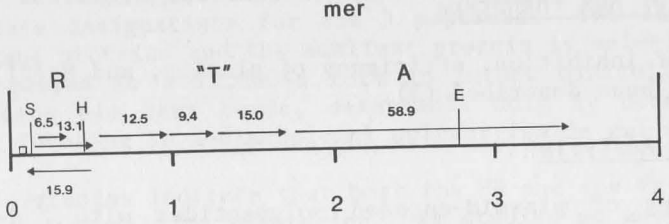

Fig. 1. Structure of the mer operon of the IncFII plasmid NR1. Distances are in kilobase pairs (kb). The small open box near the left end represents the left inverted repeat of Tn21 (8). Restriction sites indicated are EcoRI (E), HincII (H), and StuI (S). Sizes of open reading frames are indicated in kilodaltons (kd). Direction of reading frame is indicated by arrowhead.

the merR function. Two open reading frames read from left to right. The shorter of these would encode a peptide of 6,457 d and is not preceded by a Shine-Dalgarno sequence. The larger reading frame would encode a peptide of 13,139 d. There is a good Shine-Dalgarno sequence preceding this peptide, but it is 19 bp before the AUG, whereas effective translational start signals are typically found 8-9 bp before the AUG. The third possible open reading frame in the merR region lies on the complementary strand and reads towards IS1. This polypeptide would be 15,907 d and is preceded by a good Shine-Dalgarno sequence. Unlike the other 2 possible merR proteins, this one is also preceded by several good Pribnow-box-like sequences in the AT-rich region surrounding the HincII site. All 3 proteins are relatively basic and hydrophilic. Each of the 2 smaller predicted peptides would have 2 cysteines and the largest would have 4 cysteines (2).

We have previously shown that there is a merR-dependent, $HgCl_2$-inducible promoter (5) which maps in the vicinity of the HincII site. This is the least GC-rich region of the operon and contains several possible Pribnow-box (22) sequences. Restriction enzyme analysis (Ref. 5 and data not shown) indicates that this promoter directs transcription in a right direction, consistent with its being the promoter for the structural genes of the operon. The possibility that it also directs transcription to the left, i.e., that it also contains a promoter for the putative merR protein of 15,907 d encoded on the complementary strand (described above) has not been eliminated.

A transcript beginning in the promoter region and reading right would encode 4 polypeptides which we believe are the structural genes for the Hg(II) uptake system and the HR. The first open reading frame is preceded by a good Shine-Dalgarno sequence and would specify a peptide of 12,391 d. The protein has a very basic estimated isoelectric point and also has 4 cysteines. Hydropathicity analysis shows it is extensively hydrophobic (2). The second open reading frame in the Hg(II) uptake system region begins almost immediately after the first and is preceded by a Shine-Dalgarno sequence. It specifies a peptide of 9,429 d with 2 cysteines and a similarly basic isoelectric point. This peptide is largely hydrophilic except for its amino-terminus which consists of a short positively charged region followed by a sizeable ($\sim$ 15 residues) hydrophobic region (2). The third open reading frame in this region is preceded by a Shine-Dalgarno sequence and specifies a peptide of 14,965 d which has 4 cysteines and 6 methionines. It also has a basic estimated isoelectric point and is extensively hydrophobic (2).

The fourth open reading frame specifies the Hg(II) reductase of molecular mass 58,912 d. It is preceded by a Shine-Dalgarno sequence. Its estimated isoelectric point is slightly higher than the

measured value (15). It is a moderately hydrophilic protein with no extensive runs of hydrophobicity (data not shown). The protein sequence is 90% homologous to that of the HR from Tn501, with the only major discontinuity between the 2 proteins occurring in residues 70-77. However, the DNA sequences of the 2 HR genes are 60-70% homologous except for short stretches of 100 bp or less (2).

The overall GC content of the region sequenced is 63.4% with a marked gradient of GC content. The left end, up to the HincII site is 58.3% GC. The region encoding the Hg(II) uptake proteins is 61.5% and the HR structural gene is 65.2%.

Visualization of the merR Gene Product

We wished to determine which of the possible reading frames in the merR region is in fact the correct one. Since we have never seen a protein encoded by the merR cistron using the minicell system (3,14,15), to optimize expression of proteins encoded by this cistron we used the plasmid pRR134 (18) and recloned the NR1 EcoRI fragment which it carries into the regulated, high expression vehicle, pλ8 (Fig. 2). This EcoRI fragment of NR1 contains, in addition to merR, the "merT" peptides and the amino-terminal two-thirds of the HR.

The construction of the plasmids pJJ77 and pJJ80 is outlined in Fig. 2. They were designed to place the merR region in both orientations relative to the λP_L carried by pλ8. The advantage of this particular vehicle is that P_L is oriented such that it directs transcription of the β-lactamase gene (bla) of pBR322 (24). Thus the appearance of β-lactamase can be used as a diagnostic for the effective transcription of genes cloned into the sites between P_L and the bla locus. Each of these derivatives complements merR mutations as assessed by inhibition zone, efficiency of plating, and HR enzyme assay (data not shown). When expression from P_L is induced by incubation at 42°C neither pJJ77 nor pJJ80 directs the synthesis of any polypeptides (other than an N-IS1 fusion peptide, Fig. 3, fourth lane) which are not also seen in a strain carrying the vehicle pλ8 alone (Fig. 3). Thermoinducible synthesis of β-lactamase and its precursor is evident, indicating that the temperature induction worked. We discuss the implication of these results below.

DISCUSSION

1.) The HR (merA) is the largest predicted polypeptide encoded by the operon in NR1 and it contains 564 amino acids compared to 561 for the HR of Tn501 (6). While the polypeptide sequence of these 2 proteins is quite similar, the DNA sequence shows less homology. This protein appears to be the fourth in a transcript which initi-

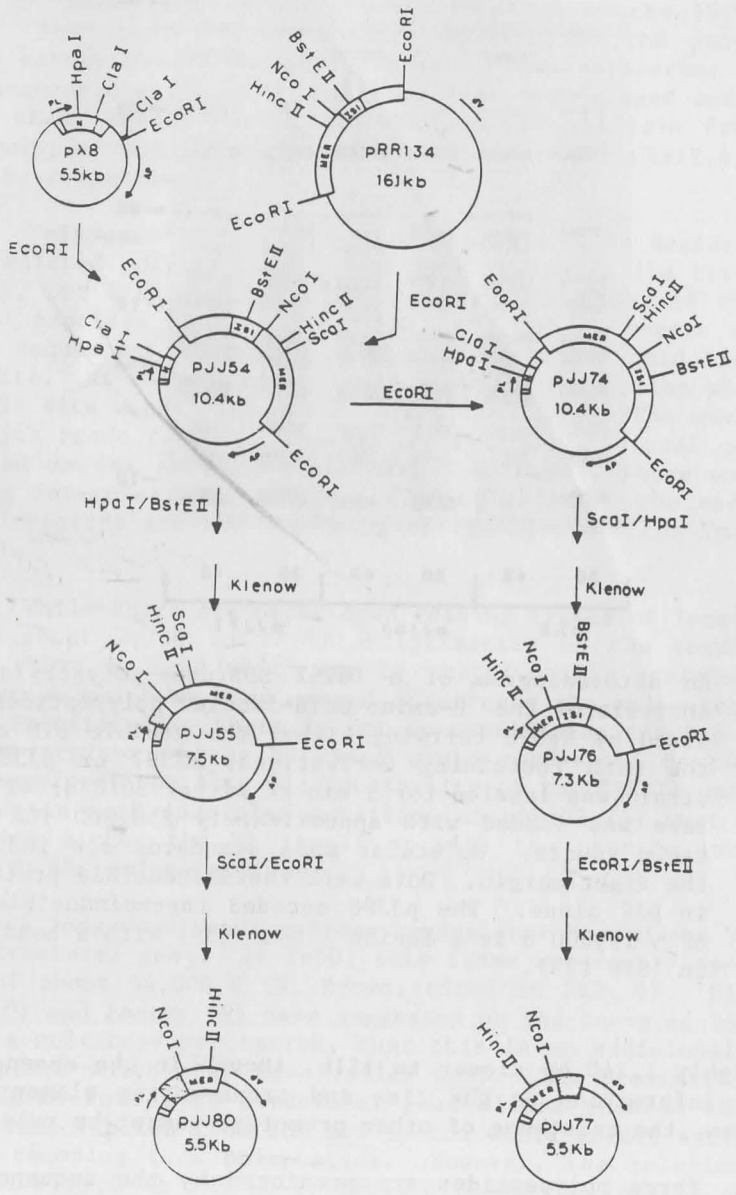

Fig. 2. Construction of the merR containing plasmids pJJ77 and pJJ80. The plasmid pRR134 carries the entire EcoRI fragment of NR1 cloned into the vehicle RSF2124 (18). This EcoRI fragment includes the merR cistron, the "merT" region and part of the HR structural gene (merA). The high expression vehicle pλ8 is described briefly in the text and in detail in Reed (24).

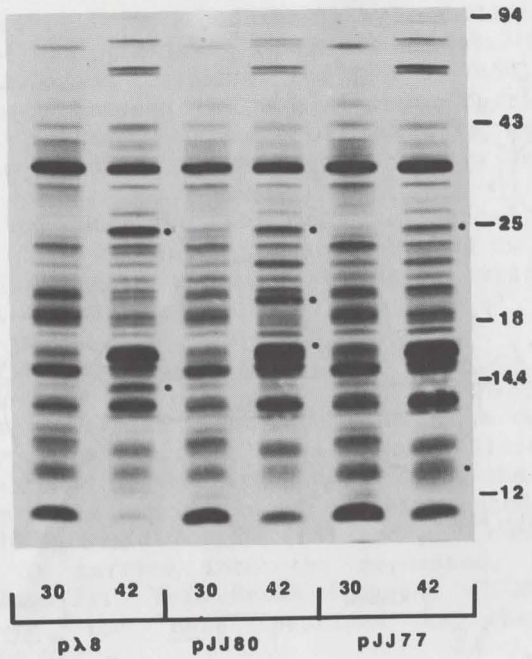

Fig. 3. An autoradiogram of a 16.5% SDS/urea-polyacrylamide gel analysis of the ^{3}H-amino acid-labeled polypeptides synthesized by N4830 carrying either the vehicle pλ8 alone, or the merR containing derivatives, pJJ77 or pJJ80. Each strain was labeled for 5 min at either 30°C or 42°C. Each lane was loaded with approximately 250,000 TCA precipitable counts. Molecular mass standards are indicated in the right margin. Dots mark thermoinducible proteins seen in pλ8 alone. The pJJ80 encoded thermoinducible peptide of ∿ 19,000 d is a fusion λ N of (39) with a heading frame in IS1b (35).

ates roughly 1,240 bp closer to IS1b, though in the absence of definitive information on the cis- and trans-acting elements of mer regulation, the existence of other promoters cannot be ruled out.

2.) Three polypeptides are predicted by the sequence in the region previously referred to as "merT." The 2 larger reading frames correspond quite well in size to the 15,000 and 14,000 d mer peptides previously described and their extensive hydrophobicity is consistent with their being intrinsic membrane proteins. Given the limits of precision of gel electrophoresis in the low molecular mass range, the smallest of these 3 predicted peptides is very likely the 13(12),000 d peptide. The amino-terminus of this predicted peptide has many features of a signal sequence, consistent with the processing and at least partial periplasmic location of the 13,000 d mer protein. The predicted amino acid sequences of the proteins all indicate rather basic isoelectric points, also consistent with previous observations (14,15). Until we have well-mapped mutations in each of these Hg(II) uptake proteins we will refrain from giving them genotypic designations and refer to them as ORF12.4, ORF9.4, and ORF15, respectively.

3.) Polypeptide analysis has not allowed us to decide which of the 3 predicted polypeptides in the merR region is the correct one. Presently, our bias is in favor of one or the other of the larger predicted peptides because, in addition to the presence of Shine-Dalgarno sequences, either of the larger peptides would include the HincII site. Ni'Bhriain et al. have shown that deletion of DNA from the HincII site right destroys merR function (20). The smaller open frame which reads from IS1b toward the operon structural genes does not extend as far as the HincII site. Moreover, there would be a very long intercistronic distance (266 bp) between the end of this predicted peptide and the beginning of the first of the "merT" peptides (2).

4.) While there are other open reading frames of lengths ranging from about 7,700 to 37,000 d (primarily on the complementary strand), there is none which can be unequivocally assigned to the merC function which we have mapped in or near the amino-terminus of the HR. Specifically, there is not an overlapping reading frame in the amino-terminus of the HR which would correspond to this function. Therefore, the loss of inducibility of the Hg(II) uptake proteins seen in merC insertion mutations of pDU202 (3) must be associated with either the HR itself or with the peptide immediately adjacent to its amino-terminus.

5.) A long open reading frame begins just beyond the 3' end of the HR structural gene. In Tn501 this frame apparently specifies a peptide of about 54,000 d (N. Brown, cited in Ref. 9). Ni'Bhriain et al. (20) and Foster (9) have suggested on the basis of Tn5 insertions in a multicopy mer operon, that this is an additional cistron of the operon which they have called merD. The proximity of this coding region to the HR structural gene almost insures that read-through transcription from the mer operon would effect synthesis of the mRNA encoding this polypeptide. However, the relationship of the putative merD to the Hg-resistance phenotype remains questionable since reduction of the copy number of the Tn5 merD mutants restored the resistant phenotype (20). In addition, deletions which remove everything to the right of a HincII site (approximately 80% of the reading frame) have no effect on the Hg-resistance phenotype of the multicopy plasmid (20). We also find that removal of the DNA right from a StuI site (∿ 50% of the 54,000 d predicted peptide) has no effect on the expression of Hg resistance by a multicopy derivative. We suspect that the Hg^{s} phenotype associated with Tn5

insertions in this region is the result of Hg-inducible overexpression of Tn5-encoded proteins which leads to instability of the multicopy plasmid. The mer region is known to have strong Hg-inducible transcriptional activity (1).

We are currently making use of the DNA sequence information for mRNA mapping, and for in vitro, synthetic linker-directed mutagenesis and translational fusion construction to confirm and extend the observations reported here.

ACKNOWLEDGEMENTS

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ANALYSIS AND MANIPULATION OF PLASMID-ENCODED PATHWAYS FOR THE CATABOLISM OF AROMATIC COMPOUNDS BY SOIL BACTERIA

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INTRODUCTION

Soil and water bacteria in general, and members of the genus Pseudomonas in particular, exhibit a fascinating wealth of exotic properties not found in commensal and parasitic bacteria, such as Escherichia coli. They are, for example, able to degrade and use as sources of carbon and energy a wide range of organic compounds, including some that are quite noxious and otherwise biocidal (e.g., phenol). Moreover, soil bacteria are able to evolve rapidly new metabolic activities in response to changes in environmental conditions, e.g., to evolve new pathways for the degradation of synthetic industrial compounds (xenobiotics) such as pesticides, that are newly applied to the environment (1). The rapidity of evolution of new pathways is particularly surprising because many pathways, e.g. those for the catabolism of aromatic hydrocarbons, involve a large number of enzymatic steps and often more than twice that number of polypeptides (2). It is hardly surprising, therefore, that many catabolic pathways are specified by plasmids (3), elements known to be instrumental for rapid genetic change in bacteria (4).

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Soil bacteria generally live and grow under adverse conditions and nutrient concentrations are frequently extremely low; such bacteria are therefore expected to dispose of a highly regulated metabolism and an efficient use of energy. The complexity of the catabolic pathways implies, therefore, a sophisticated system of regulation to control the expression and to accomplish coordination of catabolic activities. Moreover, although catabolic activities of soil bacteria are very diverse, degradative routes are convergent and lead to a rather limited number of common intermediates that are then degraded by central pathways. The existence of pathway networks thus implies a high degree of integration and a subtle interplay of regulators and inducers for segments of pathways.

Our objectives are to analyze the genetic and biochemical organization of selected plasmid-encoded pathways and to characterize genetic and biochemical strategies utilized by soil bacteria to evolve new catabolic pathways. This, in turn, involves a study of the regulatory networks that control the expression of catabolic pathways. It is our ultimate goal to utilize this knowledge in order to effect evolution in vitro, i.e., to evolve new catabolic pathways, and to increase the activities of existing pathways. We are particularly interested in evolving pathways for the degradation of recalcitrant aromatics, especially xenobiotics, because this will provide important information on the biochemical basis of recalcitrance and on microbial strategies to overcome recalcitrance. An important aspect of this program will be the exploration of physiological limits to the evolution of new catabolic routes and the increase in global activities of catabolic pathways.

We have chosen as model systems for our studies the TOL plasmid specified pathway for the degradation of toluene, xylenes and related aromatic hydrocarbons, because of the considerable amount of biochemical data available on the pathway and the particular interest of certain pathway enzymes, and the pJP4 plasmid-specified pathway for the degradation of 3-chlorobenzoate and 2,4-dichlorophenoxyacetic acid, because halogenated organics constitute one of the largest group of xenobiotic compounds released into the biosphere and contain a range of both toxic and recalcitrant compounds (5,6).

CATABOLIC PATHWAY SPECIFIED BY THE TOL PLASMID pWWO

TOL plasmid pWWO (7,8), and its Tn*401* transposon derivative pWWO-161 (9), specify a pathway for the catabolism of toluene, xylenes and related hydrocarbons (10; Fig. 1). The pathway involves successive oxidations of the side chain to give benzoate or methyl benzoate (upper pathway), followed by dioxygenation of the aromatic ring to catechol or methyl catechol, *meta* (extradiol) cleavage of the ring to produce a semialdehyde, and degradation of the semialdehyde to central metabolites (*meta*-pathway or lower pathway). The pathway branches at the ring cleavage product and reconverges at 2-oxopent-4-enoate; the ring cleavage products formed from benzoate and *p*-toluate are channelled into the 4-oxalocrotonate branch whereas the product formed from *m*-toluate is metabolized by the hydrolytic branch (11).

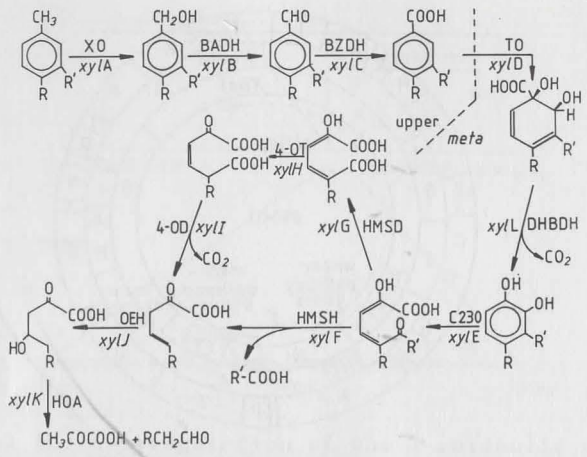

Fig. 1. TOL plasmid-specified pathway for the degradation of toluene and xylenes. Enzyme abbreviations: XO, xylene oxygenase; BADH, benzylalcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; TO, toluate/benzoate dioxygenase; DHBDH, dihydrodihydroxybenzoate dehydrogenase; C230, catechol 2,3-dioxygenase; HMSH, hydroxymuconic semialdehyde hydrolase; HMSD, hydroxymuconic semialdehyde dehydrogenase; 4-OT, 4-oxalocrotonate tautomerase; 4-OD, 4-oxalocrotonate decarboxylase; OEH, 2-oxopent-4-enoate hydratase; HOA, 2-oxo-4-hydroxy-pentonate aldolase. Initial compounds: R,R' = H: toluene; R = H, R' = CH_3: *m*-xylene; R = CH_3, R' = H: *p*-xylene. *xylA* to *xylK*: genes for the pathway enzymes (13,14).

By means of transposition mutagenesis of the TOL plasmid, and the cloning of TOL plasmid DNA segments, the catabolic genes have been localized to 2 distinct blocks, one for upper pathway enzymes and one for lower pathway enzymes (9,12; Fig. 2a). The 2 gene blocks are separated by a DNA segment about 10 kb in length. The genes of the upper pathway enzymes are organized in an operon, having the order promoter-*xylC*-*A*-*B* (13; Fig. 2b), as are the genes of the lower pathway, which have the order: promoter-*xylD*-*L*-*E*-*G*-*F*-*J*-*I*-*H* (14; Fig. 2c). Two gaps exist in the current lower pathway gene map and at least 1 gene, *xylK*, remains to be localized.

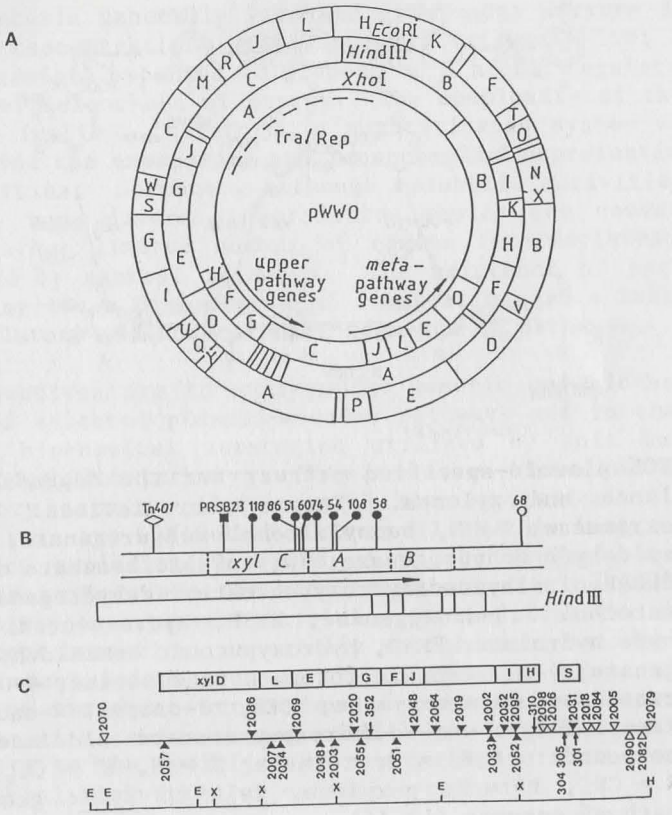

Fig. 2. The TOL plasmid pWW0 and maps of the catabolic genes.

A: Restriction endonuclease cleavage map of pWW0. Tra/Rep indicates the region of the plasmid essential for its autonomous replication and conjugal transfer ability.

B: Map of the upper pathway operon. The Tn401 and the Tn5-68 mutations do not influence expression of the operon, whereas the filled square and circles indicate insertion mutations that prevent expression of one or more structural genes. This map is taken from Ref. 13.

C: Map of the lower pathway operon. Open triangles indicate insertions that do not influence expression of the operon whereas filled triangles indicate insertions that prevent expression of 1 or more structural genes. This map is taken from Ref. 14. E, H, X: cleavage sites for EcoRI, HindIII, and XhoI, respectively.

The designations of genes of the TOL plasmid catabolic operons are explained in Fig. 1.

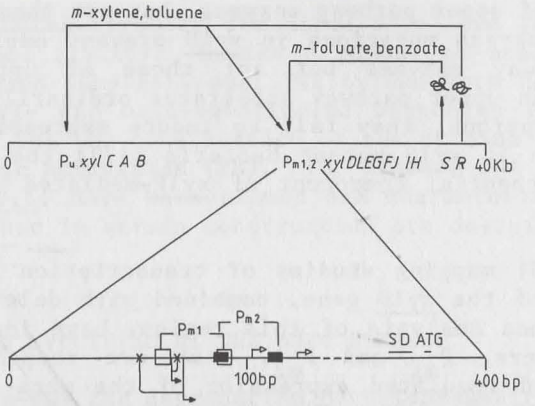

Fig. 3. Model for the regulation of the 2 catabolic operons of the TOL plasmid pWW0 and structure of the meta-cleavage pathway operon promoters.

The proposed regulatory circuits are taken from references 15 and 17. P_u, P_{m1} and P_{m2} are the promoter of the upper pathway operon and the 2 overlapping promoters of the lower pathway operon, respectively. In the expansion, the -35 and -10 regions of P_{m1} (open boxes) and P_{m2} (filled boxes) are shown, as are their corresponding transcription initiation start sites (open arrows). SD and ATG indicate the locations of the ribosome binding site and the initiation codon of the first gene of the operon.

Point mutations leading to constitutive expression of the operon are indicated by crosses, and the new transcriptional start sites associated with these mutations are shown by filled arrows.

Toluene and xylenes, and their alcohols, act as inducers and provoke synthesis of all enzymes of the pathway, whereas benzoate and toluates induce the synthesis of the enzymes of the lower pathway. The pathway is therefore organized into 2 functional parts, the conversion of the hydrocarbons to the carboxylic acids, and the conversion of the carboxylic acids to central metabolites, and this biochemical organization is reflected in the genetic organization of the pathway. It was proposed some years ago that a regulator, the product of the xylS gene, is responsible for controlling expression of genes for enzymes of the lower pathway, whereas another regulator, the product of the xylR gene, is responsible for controlling expression of genes of both parts of the pathway (15; Fig. 3). The xylS and xylR genes have now been localized downstream of the lower pathway operon (16,17; Fig. 3). Mutations in xylR prevent expres-

sion of genes of upper pathway enzymes, but not those of lower pathway enzymes, whereas mutations in xylS prevent expression of genes of lower pathway enzymes but not those of upper pathway enzymes. Although upper pathway substrates ordinarily induce expression of both operons, they fail to induce expression of the meta-cleavage operon in xylS mutant bacteria (17); the xylS product is therefore an essential component of xylR-mediated induction of the meta-operon.

Nuclease S1 mapping studies of transcription initiation sites just upstream of the xylD gene, combined with deletion and polynucleotide sequence analysis of this region, have identified 2 overlapping promoters, P_{m1} and P_{m2}, that are responsible for xylS product-mediated regulated expression of the meta-cleavage pathway operon genes (18; Fig. 3). Mutant plasmids that express constitutively the meta-pathway operon have been isolated and some of their mutations characterized; most were shown to have created new, non-regulated promoters within or just upstream of the native promoters (18). The native promoters are structurally distinct from typical E. coli promoters, which is consistent with the poor expression of TOL plasmid genes in E. coli (9,18,19, 20,21). Moreover, a range of levels of activity in E coli was observed with the constitutive TOL plasmid promoters (18), which is further evidence that promoter structure is the major factor determining differences in efficiency of expression of TOL genes in Pseudomonas and E. coli.

These studies strongly suggest that the xylS gene product is a positive regulator which acts at least partially at the level of transcription, and whose activity is essential for expression of the lower pathway enzyme genes. The xylR product would also seem to be an essential positive regulator.

Several of the TOL plasmid-specified enzymes have useful properties. For example, the ring-cleavage enzyme, catechol 2,3-dioxygenase (C230), which converts the colorless substrate catechol to the yellow-colored product 2-hydroxymuconic semialdehyde, is of considerable utility as an indicator enzyme. Its activity is readily and precisely measured by a simple photometric assay (22) and, most importantly, bacterial colonies containing the enzyme turn bright yellow when sprayed with catechol (9,19). The gene for C230, xylE, whose polynucleotide sequence has been determined (23,24), is expressed in a wide range of bacteria, including Gram positives (23), and may therefore be used for a variety of genetic manipulations, such as the isolation of promoter-containing DNA fragments (23). The xylE gene thus represents a valuable addition to the limited number of currently available indicator genes such as lacz, whose products can be detected by simple color changes.

The xylD and xylL genes encode toluate 1,2-dioxygenase and dihydrodihydroxybenzoate dehydrogenase, respectively, which convert

benzoate and toluates first to the dihydrodiol and then to catechol or methyl catechol (25,26). Unlike a number of analogous enzymes, they have a relaxed substrate specificity and form catechols from a variety of methyl- and halo-substituted benzoates (27). They are therefore useful for the construction of new strains exhibiting wide spectrum catabolic activities (28). TOL plasmid DNA segments carrying xylD or xylD,L, have been cloned and characterized; their features and their use in strain construction are described below.

CATABOLIC PATHWAY SPECIFIED BY THE pJP4 PLASMID

Two pathways for the degradation of haloaromatics have received considerable attention in recent years. The biochemistry of the modified ortho-cleavage pathway of Pseudomonas sp. B13 (WR1) for the degradation of 3-chlorobenzoate (3CB) is particularly well characterized (29,30) but its genetics have proven to be somewhat refractory. The strain contains a large plasmid, pWR1, which when transferred to other strains of Pseudomonas confers upon them the ability to utilize 3CB as sole source of carbon and energy. A closely-related plasmid, designated pAC25, has been identified in a $3CB^+$ isolate of P. putida (31). A number of $3CB^-$ transposon mutant derivatives of WR1 have been isolated and partially characterized (A.J. Weightman and M. Weisshaar, unpub. data), but the difficulty of isolation of pWR1 plasmid DNA has impeded their analysis.

A related pathway for the degradation of chloroaromatics that is less well characterized biochemically, but more amenable to genetic analysis, is the pathway for the catabolism of 3CB and 2,4-dichlorophenoxyacetic acid (24D) specified by Alcaligenes eutrophus JMP134 (1,32). In this case, crucial catabolic enzymes are encoded by the readily-isolated, broad host range P1 group plasmid pJP4.

The pathway for 24D degradation (Fig. 4) consists of removal of glyoxylic acid to form 2,4-dichlorophenol, followed by hydroxylation of the ring to form 3,5-dichlorocatechol, dioxygenation of the dichlorocatechol to form 2,4-dichloromuconate, cycloisomerization and spontaneous elimination of the chloro group at carbon atom 4, to form 4-carboxymethylene-2-chlorbut-2-ene-4-olide (2-chlorodienelactone), and hydrolysis to form 2-chloromaleylacetate. Chloromaleylacetate is further metabolized to 3-oxoadipate which is then metabolized to Krebs cycle intermediates (33,34).

A number of Tn5 and Tn1771 transposon insertion mutant derivatives of plasmid pJP4 have been obtained and characterized and 5 plasmid catabolic genes tfdB-E have been identified (35; Fig. 5). Genes tfdC-F form 1 operon whereas tfdB forms a separate operon. The enzymes encoded by four of these genes have been identified, i.e.: tfdB, 2,4-dichlorophenol hydroxylase, tfdC, chlorocatechol

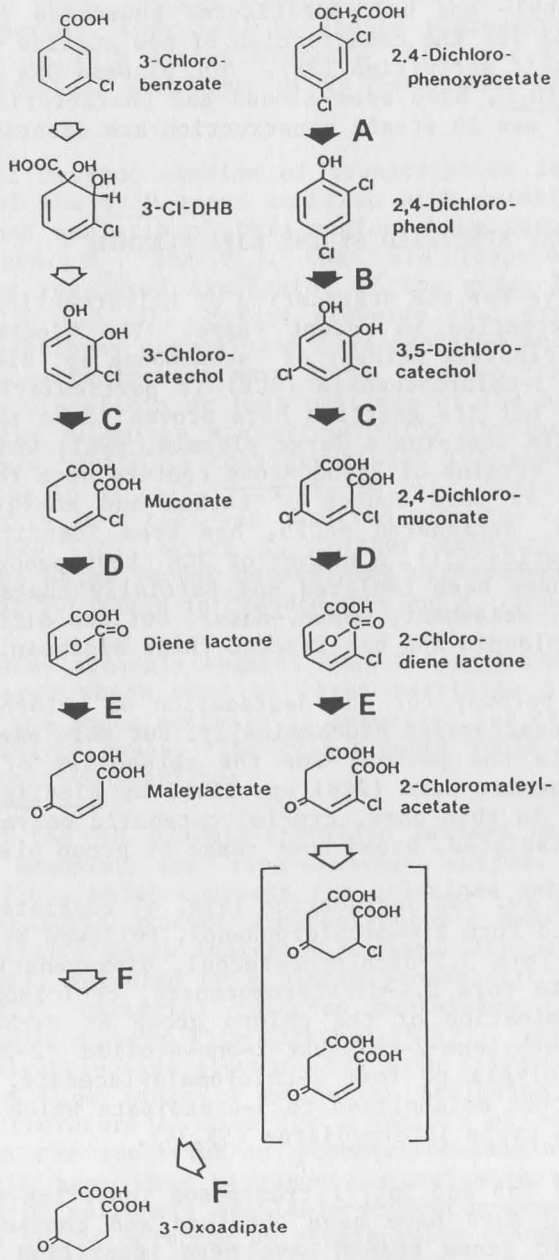

Fig. 4. Proposed pathways for the degradation of 3-chlorobenzoate (3-chlorocatechol branch) and 2,4-dichlorophenoxyacetic acid in Alcaligenes eutrophus JMP134.

The letters in bold type indicate the pJP4 plasmid-carried catabolic genes tfdA,B,C,D,E, and F, which specify the enzymatic steps indicated by filled arrows. The two initial steps in the degradation of 3-chlorobenzoate are specified by chromosomal genes. The nature of the tfdF gene product is not yet established but suspected of being a trans-chloridiene lactone isomerase (see text).

1,2-dioxygenase; tfdD, chloromuconate cycloisomerase; tfdE, dienelactone hydrolase. The inability of plasmid-free derivatives of JMP134 to attack 24D suggests that the tfdA gene, which encodes the first enzyme of the pathway 24D monooxygenase, is plasmid borne. However, no mutants defective in this enzyme have thus far been obtained, and hence its location remains to be established. On the other hand, a fifth plasmid gene, tfdE, that encodes a step in 24D degradation (perhaps trans-chlorodiene-lactone isomerase) not thus far defined, has been localized on pJP4 (Fig. 5). Mutations in tfdC, D and E prevent growth of JMP134 on 3CB. Mutations in tfdF severely retard growth of JMP134 on 24D but not on 3CB. Introduction of the cloned tfdC, D, E genes into a pJP4-cured derivative of JMP134 enables the derivative to grow on 3CB, but not 24D. These results indicate that 24D is degraded to 3-oxoadipate by plasmid-encoded enzymes, whereas 3CB is transformed by enzymes encoded by the chromosome to chlorocatechol which is then degraded to 3-oxoadipate by the plasmid-encoded 24D pathway.

All identified catabolic genes of plasmid pJP4 have been localized to EcoRI fragment B. This fragment has been cloned in the pKT231 broad host range cloning vector plasmid in E. coli and subsequently transferred to a pJP4-cured derivative of JMP134 by RP4-mediated mobilization (35). Transconjugants containing pKT231-pJP4 EcoRI-B hybrid plasmids were able to grow on 24D and 3CB as sources of carbon and energy. The $24D^+$ and $3CB^+$ phenotypes could not, however, be maintained in the absence of continued selection pressure.

MANIPULATION OF CATABOLIC PATHWAYS BY MEANS OF CLONED GENES

An important potential use of a detailed biochemical and genetic characterization of a catabolic pathaway is the experimental manipulation of its activities. Manipulations of a catabolic pathway may be effected in order to accomplish a number of objectives, including:

(1) a vertical expansion of the pathway, i.e., an increase in

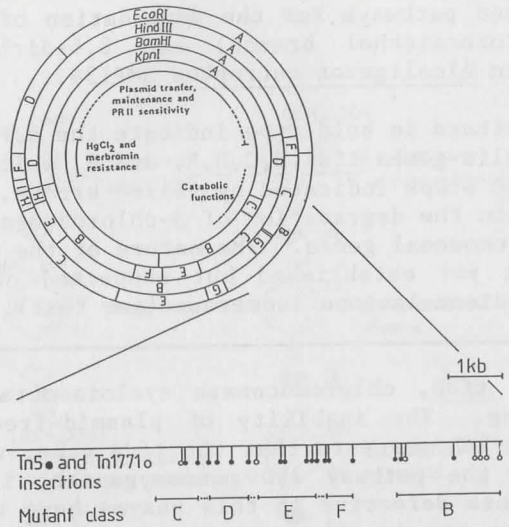

Fig. 5. Physical and genetic map of the pJP4 plasmid.

its length, accompanied by a corresponding increase in the number of compounds it can process (e.g., addition of genes encoding the conversion of chloronaphthalene to chlorocatechol to genes of a chlorocatechol pathway; see below);

(2) a horizontal expansion of a pathway, i.e., effecting a reduction in its specificity to enable degradation of a larger series of substrate isomers and analogues (Ref. 21, 25; see below);

(3) an increase in its activity;

(4) an alteration of the end product of the pathway, to provoke accumulation of a specific intermediate or to channel it into another pathway (26);

(5) an alteration in its regulation, e.g., in order that it be expressed constitutively (see above) or that new compounds act as inducers.

Gene cloning procedures are ideally suited for effecting the first four types of manipulation (21), and may be very useful for the last, although here an in vivo procedure may be the preferred choice initially. The manipulation of catabolic pathways by gene cloning methods has the major advantage over traditional methods of in vivo manipulation in that defined segments of DNA are used to effect predictable changes. Moreover, where supplementary genetic adjustments occur in vitro during a manipulation, they are more readily characterized at the molecular level. Perhaps most importantly, multiple changes can be effected either stepwise with high efficiencies or in a single step; classical procedures for effecting multiple genetic changes are invariably inefficient and require complicated and lengthy selection regimes.

We are currently exploring possibilities for the directed manipulation in vitro of catabolic pathways and are focusing our efforts on pathways for the degradation of aromatics and haloaromatics. Of crucial importance for these manipulations is the fact that many such pathways converge at a few common intermediates, such as catechol, or derivatives of catechol, which are the substrates for cleavage of the aromatic ring. Catechol may be subjected to ortho (intradiol) or meta (extradiol) cleavage (Fig. 6). The nature of the mode of ring fission and the corresponding pathway to tricarboxylic acid cycle intermediates depends upon the nature of the catechol derivative: catechol itself can be degraded by either pathway; on the other hand, methyl catechols are generally degraded by the meta pathway whereas chlorocatechols are usually degraded by a modified ortho-pathway. Chlorocatechols are in fact readily cleaved at the meta position but the resulting ring cleavage product either inhibits irreversibly the ring cleavage enzyme or is further metabolized to chlorosubstituted dead-end metabolites (36). Pathway

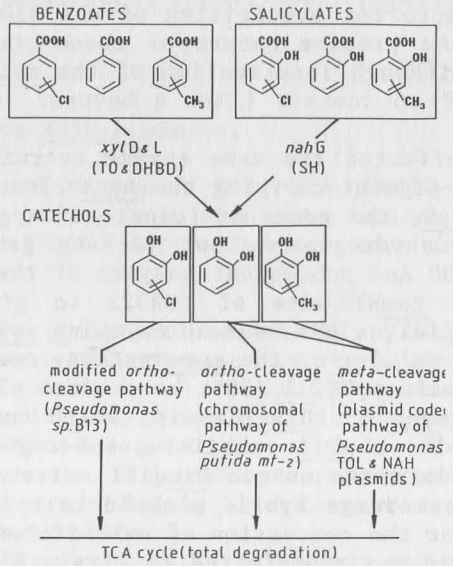

Fig. 6. Conversion of substituted benzoates and salicylates to catechols by broad specificity enzymes of cloned genes, and subsequent pathway for dissimilation of the catechols.

manipulation strategies must therefore take into account the need to metabolize a substrate to one of a few central intermediates, the need for the availability of an appropriate pathway for the further degradation of the selected intermediate, and the need to eliminate any pre-existing non-productive pathway for that compound.

Horizontal expansions of a 3CB pathway

Pseudomonas sp. B13 (29) degrades 3-chlorobenzoate, but not 4-chlorobenzoate (4CB) or 3,5-dichlorobenzoate (35DCB), owing to the fact that the first enzyme of the pathway, benzoate 1,2-dioxygenase, has a narrow substrate specificity and thus a low affinity for the latter 2 analogues (27). As indicated above, the substrate specificity of the equivalent enzyme encoded by the TOL plasmid of P. putida mt-2 is broader and the enzyme is able to transform 3CB, 4CB and 35DCB.

In an early experiment (28) to examine the feasibility of recruiting the TOL plasmid enzyme into the 3CB pathway, the TOL plasmid was transferred into strain B13. Transconjugants selected for their ability to grow on m-toluate were not able to utilize either 4CB or 35DCB as growth substrates. Mutant derivatives able to grow on 4CB could, however, be isolated from such transconjugants, as indeed could derivatives able to degrade 35DCB, although at lower frequencies. Apparently, therefore, recruitment of the TOL plasmid broad specificity enzymes into the 3CB ortho-pathway required at least one genetic change for acquisition of the 4CB$^+$ phenotype and at least one more for acquisition of the 35DCB$^+$ phenotype. As indicated above, the precise nature of these changes could not be readily analyzed although inactivation of the meta-cleavage pathway was observed (28,37).

We have now effected the same enzyme recruitment using cloned genes (25). A DNA segment carrying the meta-cleavage pathway operon promoters and xylD,L, the genes of toluate dioxygenase and dihydrodihydroxybenzoate dehydrogenase, but lacking genes for the meta-cleavage enzyme C230 and subsequent enzymes of the meta pathway, was inserted into the BamHI site of pBR322 to give plasmid pPL401 (Fig. 7). In parallel, a DNA segment carrying xylS, the gene encoding the positive regulator of the meta-pathway operon, was cloned in the XhoI site of vector pKT231 (38), to produce plasmid pKT570. The large HindIII fragment of this plasmid, which contains most of the vector sequences plus a xylS containing sub-segment of the insert, was then inserted into the unique HindIII site of pPL401, to give pPL403, a broad host range hybrid plasmid carrying regulated genes encoding enzymes for the conversion of substituted benzoates to catechols. This plasmid was transferred to strain B13 but was unstable in this host. Stable derivatives were, however, readily selected and structural analysis of a hybrid plasmid, pPL403-1, from one derivative suggested that stability was gained as a result of a

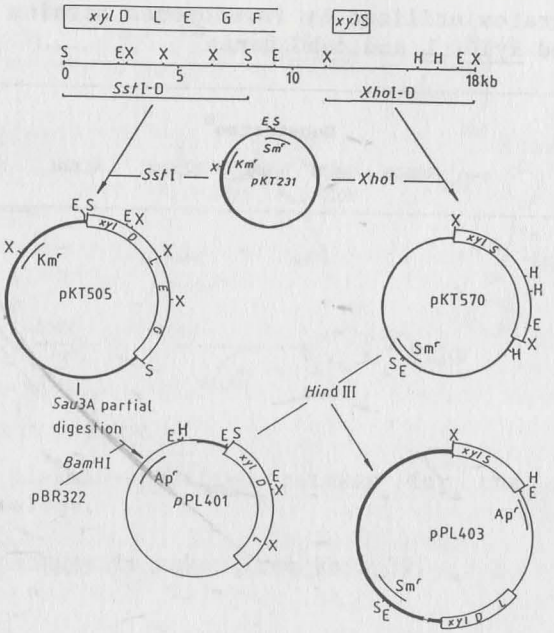

Fig. 7. Construction of a broad host range plasmid carrying the TOL plasmid xylD, L, S genes.

Abbreviations: S,E,X,H, cleavage sites for restriction endonucleases SstI, EcoRI, XhoI, HindIII, respectively.

deletion event that removed a small segment of vector DNA, located just upstream of the xylD,L operon.

When transferred to strain B13, the pPL403-1 plasmid extended the catabolic range of transconjugants to include 4CB, but not 35DCB (Tab. 1). However, 35DCB$^+$ derivatives of such transconjugants were readily isolated.

Analysis of the ability of mutants of pPL403, deleted of various segments of insert DNA, to give rise to 35DCB$^+$ derivatives showed that, whereas acquisition of the xylD gene alone permitted B13 to catabolize 4CB, acquisition of both xylD and xylL were necessary for the isolation of 35DCB$^+$ derivatives of B13. These results tentatively suggest that the dihydrodihydroxybenzoate dehydrogenase of the 3CB pathway of strain B13 is able to process the 4CB metabolite but not the 35DCB metabolite.

Thus expansion of the 3CB pathway to include 4CB by acquisition of the cloned xylD gene required no supplementary genetic change; expansion of the pathway to include 35DCB by acquisition of the

Tab. 1. Substrates utilized by Pseudomonas strains containing the cloned xylD, L and nahG genes[a]

Pseudomonas strain	Substrates[b] ben	3CB	4CB	SAL	3CSAL	4CSAL	5CSAL
P.putida KT2442	+	-	-	-	-	-	-
KT2442 (pPL300-1)	+	-	-	+	-	-	-
P.sp B13	+	+	-	-	-	-	-
B13 (pPL300-1)	+	+	-	+	+	+	+
B13 (pPL403-1)	+	+	+	-	-	-	-

[a] Growth determinations were carried out on solid and in liquid media; + indicates growth, - indicates no growth.

[b] Abbreviations: ben, benzoate; 3CB, 3-chlorobenzoate; 4CB, 4-chlorobenzoate; SAL, salicylate; 3,4,5CSAL, 3-, 4-, 5-chlorosalicylate.

cloned xylD,L genes did require such a change. 35DCB is not an effective inducer of the meta-pathway operon and we are therefore currently investigating possible modifications of the meta-pathway operon regulatory system in 35DCB$^+$ derivatives, particularly the molecular changes that may have occurred in the cloned xylS gene-containing DNA fragment.

A second type of expansion of the chlorocatechol pathway of strain B13, this time to include chlorosalicylates, was accomplished by introduction of the cloned gene for a salicylate hydroxylase (25). The NAH7 plasmid of P. putida encodes all enzymes required

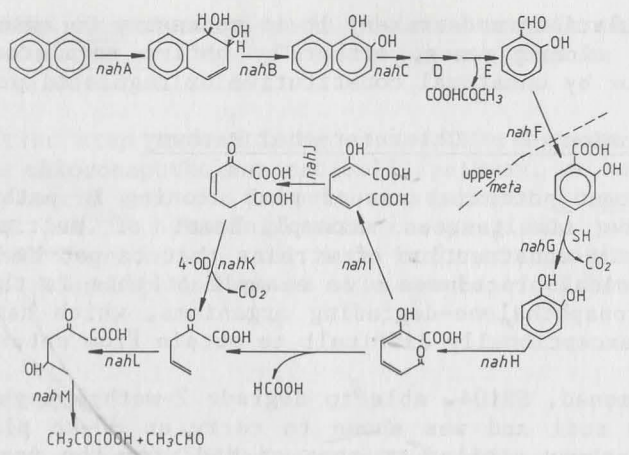

Fig. 8. NAH7 plasmid-specified pathway for the degradation of naphthalene.

This pathway is taken from Ref. 39.

for the catabolism of naphthalene via salicylate, catechol and a meta-cleavage pathway (39; Fig. 8). The salicylate hydroxylase encoded by the nahG gene of NAH7 exhibits a broad substate specificity and metabolizes salicylate, methyl- and chlorosalicylates to their corresponding catechol derivatives (25). A HindIII-generated fragment of the NAH7 plasmid that contains the nahG gene was inserted into pBR322 and the hybrid plasmid thereby constructed, pPL299, was fused to the pKT231 vector through their unique EcoRI cleavage sites, to produce pPL300. This hybrid was transferred to Pseudomonas bacteria but was found to be unstable. Stable derivatives were readily isolated and found to have suffered a small deletion of vector sequences just upstream of the cloned nahG gene. One such deletion derivative plasmid, pPL300-1, was transferred to strain B13 which, as a result, acquired the ability to degrade and use as sole source of carbon 3-, 4- and 5-chlorosalicylate. Thus, as was the case for expansion of the 3CB pathway to include 4CB, expansion of the same pathway to include chlorosalicylates required no genetic change other than that of acquisition of the gene of the enzyme to be recruited. This is almost certainly due to an absence on the cloned DNA segments of genes for enzymes of the non-productive meta-cleavage pathway.

Synthesis of salicyate hydroxylase in Pseudomonas bacteria containing pPL300 or pPL300-1 was inducible by salicylate, indicating that the cloned HindIII fragment contains the determinant of a regulator for the nahG gene. Thus, in both types of construction, inducible expression of the cloned genes was preserved. Clearly, in order for enzyme recruitment to be effectively accomplished by the

genetic manipulations undertaken, it is necessary to assure balanced expression of cloned genes, either by native regulatory circuits (preferably) or by unnatural constitutive or regulated promoters.

Vertical Expansion of a Chloracatechol Pathway

An important potential use of gene cloning in pathway manipulation is the simultaneous accomplishment of multiple genetic changes, and the construction of strains that cannot be easily isolated by classical procedures. An example of this is the construction of chloronaphthalene-degrading organisms, which have thus far proven to be exceptionally difficult to obtain from natural sources.

A pseudomonad, SB104, able to degrade 2-methyl naphthalene was isolated from soil and was shown to carry an 80-kb plasmid which specifies a pathway similar to that of NAH7 for the degradation of naphthalene via salicylate, catechol and a meta-cleavage pathway. This plasmid, pSB104, is structurally similar though not identical to NAH7, and its catabolic pathway exhibits minor differences to that of NAH7 in terms of regulation of the pathway and the specificities and activities of certain enzymes (S. Bas, unpub. data).

Plasmid pSB104 catabolizes 2-chloronaphthalene to chlorocatechol. In order to explore the possibility of combining the chloronaphthalene to chlorocatechol pathway of SB104 with a chlorocatechol degradation pathway, an insertion mutant derivative of pSB104, containing a Tn5 insertion element in its C230 gene (i.e., a derivative

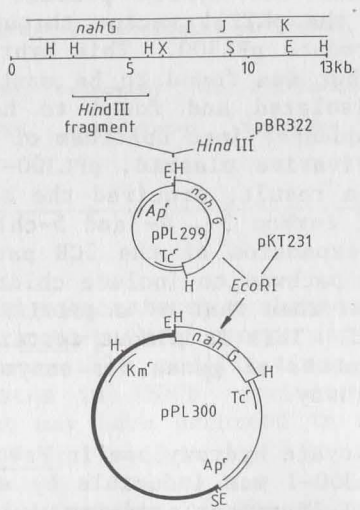

Fig. 9. Construction of a broad host range plasmid carrying the nahG gene of plasmid NAH7.

Abbreviations used are those described in Fig. 7.

blocked in the meta-cleavage pathway), was transferred by conjugation to Pseudomonas sp. B13. Transconjugants grew on 2-chloronaphthalene and released chloride.

As a first step in utilizing cloned DNA segments to construct an effective chloronaphthalene catabolic pathway, we have introduced the pSB104 plasmid into the laboratory strain of P. putida, KT2442, containing a pKT231 hybrid plasmid carrying the pJP4 genes for chlorocatechol degradation, and have isolated transconjugants able to grow on 2-chloronaphthalene as sole source of carbon and energy.

CONCLUDING REMARKS

The possibilities for the controlled, selective manipulation of microbial metabolic pathways by genetic engineering methods are enormous. Indeed, classical methods to effect such manipulations have been exploited for years to great effect in the area of secondary metabolism. Where a particular change can be selected or readily recognized, where the molecular details of the manipulation are not required, and in particular where little biochemical and genetic information on the pathway exists, a classical approach may still in most cases be the one of choice. However, where multiple changes are necessary, where no effective selection exists for the desired change, and particularly for those manipulations for which an understanding of the molecular events of the change is desired, the use of gene cloning procedures to introduce defined segments of DNA encoding just those enzymes of relevance into selected microorganisms is indicated.

The availability of broad host range cloning vectors and transposon donors (e.g., 21, 40) now permits genetic analysis and manipulation of a wide range of microbes, including those exhibiting a variety of important and interesting properties. It should be emphasized, however, that manipulation of complex pathways is most effective where extensive biochemical and genetic information already exists, as was the case for the experiments discussed here.

In this report, we have described the characterization of 2 model catabolic pathways, those of the TOL and pJP4 plasmids, and experiments in which cloned genes encoding broad specificity enzymes have been used to expand existing pathways for the degradation of chlorocatechols, and a cloned operon encoding chlorocatechol degradation has been used to create a hybrid pathway that catabolizes chloronaphthalene. These experiments have defined which enzymes were necessary for expansion for the 3CB pathway to include 4CB and 35DCB, and have indicated that a further mutation necessary for acquisition of the 35DCB phenotype may involve the regulation system for expression of the xylD,L genes.

It is to be anticipated that during the analysis of additional, in some instances unexpected, changes necessary for certain manipulations, important information on pathway functioning in vivo will be obtained, particularly on physiological and cellular parameters that constrain such manipulations, and on the regulatory circuits that assure optimal integration of the pathway under investigation with other other cellular metabolic activities.

ACKNOWLEDGMENTS

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THE PLASMID-SPECIFIED AEROBACTIN IRON UPTAKE SYSTEM OF *ESCHERICHIA COLI*

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INTRODUCTION

Plasmids and Pathogenicity

Escherichia coli is a major aerobic commensal bacterial species in the intestinal flora of a healthy individual. Some strains, however, are pathogenic for man, causing disease by a variety of mechanisms, and with a severity that ranges from the temporary, if acute, discomfort of "turista" to the chronic human tragedy of widespread infant mortality in the third world. In domestic animals, too, *E. coli* infections are of significant economic importance in terms of both lost production and cost of control and treatment.

The virulence of *E. coli* strains can in many cases be attributed to the presence of plasmids. For instance, the plasmid location of the genetic determinants of the enterotoxins, of which one, the heat labile toxin, is very closely related to the chromosome-specified cholera toxin of *Vibrio* (19,36), has been known since the pioneering studies of H. Williams Smith in the 1960s (32). Moreover, it is clear from his early work with animal pathogens (34), and has since been confirmed with enterotoxigenic isolates from human disease (11,12), that plasmid-mediated surface antigens promote bacterial colonization of the intestinal mucosa, and thereby effectively concentrate toxin action at target cells. By contrast, it has been shown relatively recently that plasmids are implicated in the virulence of enteroinvasive strains of *E. coli*, which penetrate and proliferate in the mucosal epithelium and elicit dysentery-like diarrheal disease (18).

In a search for novel plasmid-specified virulence determinants among isolates of *E. coli* from cases of bacteremia of man and a

range of domestic animals, H. Williams Smith reported that a significant proportion of strains carried plasmids specifying the antibacterial protein, colicin V (31). Furthermore, he showed that loss of such ColV plasmids by curing markedly reduced the virulence of clinical isolates in experimental infections of a variety of laboratory and domestic animals, and, conversely, that gain of a ColV plasmid by conjugation significantly enhanced the virulence of laboratory strains of E. coli (31,33). Several phenotypic properties of ColV plasmids have been proposed to contribute to this phenomenon, including increased ability to adhere to epithelial cells (9) and resistance to the bactericidal effects of serum (4). Moreover, it has been suggested tha colicin V itself might aid bacterial invasion by increasing vascular permeability or by depressing macrophage activity (25,26). Of crucial importance in the progress of extraintestinal infections, however, is the operation of a plasmid-determined high affinity mechanism for the specific active uptake of iron which allows extensive bacterial proliferation in the tissues and body fluids of an infected animal. It is this particular aspect of ColV plasmids that we discuss in detail here.

Iron and Infection

Iron is essential for microbial growth. In nature, however, iron is virtually unavailable to microorganisms because it forms insoluble aggregates at physiological pH or because it exists in complexed forms, such as hemoglobin, in animal bodies. Indeed, the level of free ferric ions in the blood and other body fluids of a healthy individual is well below that required for bacterial growth because of the presence of the iron-binding glycoproteins "transferrin" in serum and "lactoferrin" in secretions (38). Furthermore, systemic infections may provoke a host hypoferremic response in which the level of ferric ions in the serum transferrin iron pool is reduced, thereby limiting the availability of extracellular iron to invading microorganisms (38). It is to be expected, therefore, that a mechanism which permits more efficient sequestration of iron from the complexed state would be an important component in the virulence of strains of E. coli associated with extraintestinal infections. Evidence that ColV plasmids mediate such a mechanism is provided by the observation that they confer on the host bacterium a significant selective advantage for growth in iron-limited conditions, both in vitro, in media containing transferrin, and in vivo, in the bodies of experimentally infected animals (40).

ColV Plasmid-Mediated Iron Uptake

E. coli, and other enteric bacteria, secrete the catechol siderophore "enterochelin" (enterobactin) to solubilize and sequester iron in the medium and to actively transport it into the cell (29). Mutants defective in the biosynthesis of enterochelin rely on low affinity, nonspecific iron uptake from media in which iron is present at relatively high levels (13). Alternatively, some bacterial strains will take up externally supplied iron-chelating compounds such as citrate (13) or ferrichrome (22). However, the presence of a ColV plasmid allows growth of an enterochelin-deficient mutant of E. coli K-12 in iron-limited conditions in the absence of added citrate, because of an alternative mechanism for the efficient active uptake of ferric ions from the medium. Phenotypic analysis of plasmid mutants defective in iron uptake (41) suggested the secretion of an extracellular chelating compound, defined by iuc mutations, and a specific ferric-siderophore transport function, defined by the iut mutation, which, like other bacterial active transport mechanisms, requires functional tonB protein (40). The iut gene product is an outer membrane protein (M_r = 74,000) whose synthesis is induced in conditions of iron starvation (15). This protein is, fortuitously, also the receptor for cloacin DF13, a bacteriocin of Enterobacter cloacae (2,35). Thus, strains of E. coli, which are naturally insensitive to cloacin DF13, are rendered susceptible by the presence of ColV plasmids and, furthermore, mutants of the iut type are cloacin-resistant. The siderophore whose synthesis is defined by the iuc mutations was identified as aerobactin (37), a hydroxamate compound previously purified from and named for a strain of Aerobacter aerogenes (14). In addition, strains of En. cloacae (35), Shigella flexneri (27), Salmonella austin, S. memphis, and Arizona hinshawii (24) have also been shown to secrete aerobactin.

PHYSIOLOGICAL ASPECTS OF AEROBACTIN-MEDIATED IRON UPTAKE

Acquisition of Iron by Aerobactin

The association constant of ferric-aerobactin (approximately 10^{23}, Ref. 23) is many orders of magnitude lower than those of ferric-enterochelin (10^{52}, Ref. 23) and ferric-transferrin ($\geq 10^{30}$, Ref. 38). It is interesting to speculate, therefore, how such a thermodynamically inferior siderophore is able to confer significant selective advantage for growth in vivo upon bacterial cells which can also synthesize the more powerful chelator enterochelin. Exchange of iron between transferrin and aerobactin probably proceeds through the initial formation of a ternary complex in which iron is bound to both compounds (20), and in fact the rate of removal of iron from transferrin is significantly higher to aerobactin than to other hydroxamate chelators with superior affinities for ferric iron, implying that the chemical structure of the competing ligand, rather than merely its affinity for iron, plays an important role in the acquisition of iron. In this chapter, we present evidence that, in addition to particular structural features as yet undefined, aerobactin possesses physiological and regulatory properties which enhance its efficiency as a siderophore in environmental conditions of extreme iron stringency.

Intracellular Compartmentalization of Aerobactin Iron

Streptonigrin is an aminoquinone antitumor agent. It can kill bacteria by degrading the DNA, a process which depends upon the presence of ferric iron (39,42) and we have exploited its bactericidal effect as a probe of the level of free iron within bacterial cells. Figure 1 illustrates the growth curves of strains of E. coli in appropriately supplemented M9 minimal medium; cultures were inoculated with nutrient broth-grown cells, and subsequently subcultured into fresh minimal medium to maintain logarithmic growth; samples taken at 0, 3, and 6 hr were tested for sensitivity to streptonigrin.

Strain W3110 secretes enterochelin. Strain LG1315 is enterochelin-deficient (entA), but it carries plasmid ColV-K30 (37) which specifies aerobactin biosynthesis. Both strains grew at the same constant rate (doubling time, 40 min) for the duration of the experiment (Fig. 1a). Strain LG1418, which is an iuc derivative of LG1315 and so can synthesize neither siderophore, also grew well for several hours; prior growth in iron-rich medium such as nutrient broth allows accumulation of intracellular pools of iron which permit growth for several generations following subculture to iron-limited medium. Eventually, growth of LG1418 ceased, presumably when intracellular supplies of iron were depleted, although addition of sodium citrate promoted rapid restoration of active cell division due to the uptake of iron as ferric-citrate (Fig. 1a). Consistent with these proposed physiological changes is the observation that strain LG1418 became increasingly resistant to the bactericidal action of streptonigrin during progressive depletion of the intracellular iron pool (Fig. 1b-d). Sensitivity to streptonigrin was regained upon addition of citrate to the medium.

It is probable that depletion of iron pools occurs to some extent after a shift from nutrient to minimal medium even in cells that are actively taking up iron, since strain W3110, although remaining very sensitive to streptonigrin during growth due to secretion of enterochelin, did show a slight increase in resistance over the course of the experiment (Fig. 1b-d). By contrast, aerobactin-promoted growth of strain LG1315 at maximal rate was accompanied by rapid loss of streptonigrin-sensitivity, suggesting that aerobactin is in some way able to direct assimilated iron for bacterial growth, rather than, as with enterochelin and citrate, contribute it to a pool from which the rate of withdrawal for growth may be concentration-dependent.

Siderophore Activity at Low Concentrations

Although strain LG1418 synthesizes no siderophores, it can take up both aerobactin and enterochelin when supplied exogenously. Using a culture of LG1418 incubated in minimal medium to deplete intracellular iron pools (as described in Fig. 1a), we have demon-

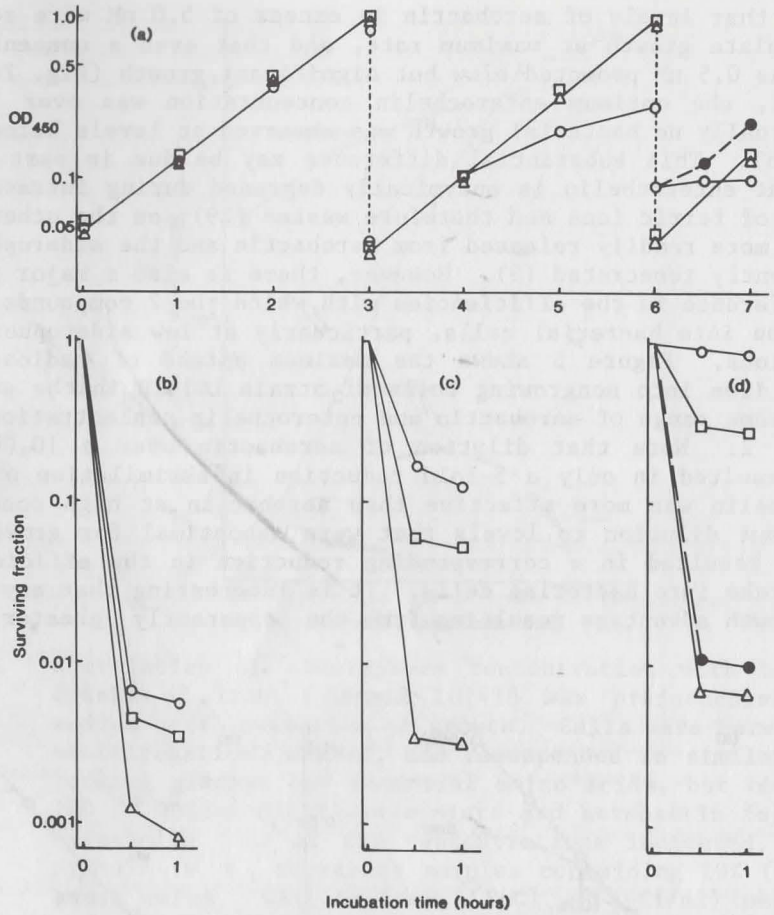

Fig. 1. Correlation of streptonigrin sensitivity with depletion of intracellular iron pools. (a) Nutrient broth-grown cells of E. coli strains W3110 (△), LG1315 (□), and LG1418 (○ ●) were washed, resuspended in M9 medium, and incubated with aeration at 37°C. Bacterial growth was followed by measurement of optical density at 450 nm, and cultures were diluted with fresh medium at 3 and 6 hr. Samples were removed for determination of streptonigrin sensitivity at time 0 hr (b), 3 hr (c), and 6 hr (d). Cells were harvested by centrifugation, washed, and resuspended in phosphate-buffered saline to an optical density at 450 nm of 0.5. Streptonigrin was added at 40 μM, and samples were taken for estimation of viable cell counts at 0, 30, and 60 min during incubation at 37°C. Sodium citrate (10 mM) was added to a portion of the LG1418 culture (●) after the second subculture, and incubated for a further 1 hr before determination of streptonigrin sensitivity.

strated that levels of aerobactin in excess of 5.0 nM were adequate to stimulate growth at maximum rate, and that even a concentration as low as 0.5 nM promoted slow but significant growth (Fig. 2a). By contrast, the optimum enterochelin concentration was over 500 nM, and virtually no bacterial growth was observed at levels below 50 nM (Fig. 2b). This substantial difference may be due in part to the fact that enterochelin is enzymically degraded during intracellular release of ferric ions and therefore wasted (29); on the other hand, iron is more readily released from aerobactin and the siderophore is subsequently resecreted (5). However, there is also a major intrinsic difference in the efficiencies with which the 2 compounds transport iron into bacterial cells, particularly at low siderophore concentrations. Figure 3 shows the maximum uptake of radioactively labeled iron into nongrowing cells of strain LG1418 in the presence of the same range of aerobactin and enterochelin concentrations used in Fig. 2. Note that dilution of aerobactin over a 10,000-fold range resulted in only a 5-fold reduction in assimilation of ^{55}Fe; enterochelin was more effective than aerobactin at high concentrations, but dilution to levels that were suboptimal for growth (see Fig. 2) resulted in a corresponding reduction in the efficiency of iron uptake into bacterial cells. It is interesting that any potential growth advantage resulting from the apparently greater effi-

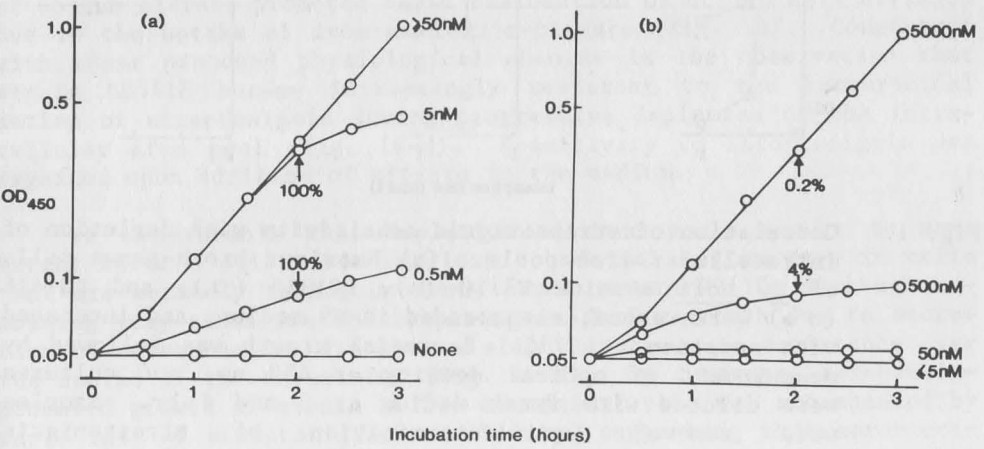

Fig. 2. Concentration optima of aerobactin and enterochelin. Strain LG1418 was preincubated in M9 medium until cessation of cell division. Aerobactin (a) or enterochelin (b) were added at the concentrations indicated, and bacterial growth was followed by measurement of optical density at 450 nm during incubation with aeration at 37°C. Percentage figures show the proportion of cells surviving streptonigrin treatment for 60 min (as described in Fig. 1) in culture samples designated by the arrows.

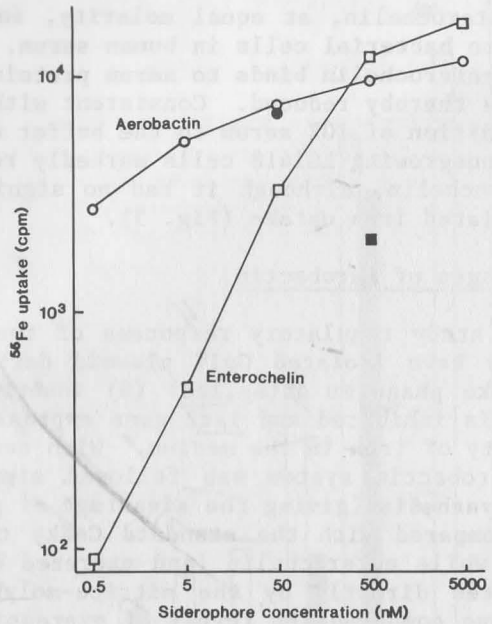

Fig. 3. Correlation of siderophore concentration with bacterial uptake of iron. Strain LG1418 was preincubated in M9 medium until cessation of growth. Cells were harvested by centrifugation, washed, and resuspended in similar medium lacking glucose and essential amino acids, but containing 100 μM sodium nitrilotriacetate and aerobactin (o) or enterochelin (□) at the concentrations indicated. Filled symbols (● ■) represent samples containing 10% (vol/vol) human serum. Carrier-free $^{55}FeCl_3$ (1 μCi/ml) was added, and suspensions were incubated with aeration for 120 min at 37°C. Cells from 5 ml of suspension were washed on membrane filters with medium containing 100 mM sodium citrate. Filters were air-dried and the level of ^{55}Fe label retained was counted; points are the average of duplicate samples.

ciency of iron uptake by high enterochelin concentrations is dissipated because assimilated iron is released to an intracellular pool. This is shown by the marked streptonigrin sensitivity of LF1418 supplied exogenously with enterochelin (Fig. 2b); even at a ferric-enterochelin concentration that is rate-limiting for growth, only 4% of the population survived streptonigrin treatment. By contrast, cells supplied with aerobactin remained streptonigrin-resistant during growth (Fig. 2a), again suggesting compartmentalization of the iron sequestered by aerobactin, rather than its contribution to a pool. Konopka et al. (20) have demonstrated that aerobactin is more

effective than enterochelin, at equal molarity, in delivering iron from transferrin to bacterial cells in human serum. This may be due to the fact that enterochelin binds to serum proteins and its siderophore activity is thereby reduced. Consistent with this is our observation that addition of 10% serum to the buffer used to determine ^{55}Fe uptake into nongrowing LG1418 cells markedly reduced the effectiveness of enterochelin, although it had no significant influence on aerobactin-mediated iron uptake (Fig. 3).

Regulatory Advantages of Aerobactin

In order to study regulatory responses of the aerobactin iron uptake system, we have isolated ColV plasmid derivatives carrying the transposon-like phage Mu dl(Ap,lac) (8) inserted so that aerobactin synthesis is inhibited and lacZ gene expression is regulated by the availability of iron in the medium. With such mutants, regulation of the aerobactin system was followed simply by measuring β-galactosidase synthesis (giving the advantage of greater sensitivity and safety compared with the standard Csáky test for hydroxamates, Ref. 10), while enterochelin (and excreted degradation products) was estimated directly by the nitrite-molybdate reagent of Arnow (1). We have compared the levels of expression of both iron-uptake systems in response to increasing iron stress exerted by adding iron or an iron chelator (α,α'-dipyridyl) to M9 medium (which itself contains approximately 2 μM Fe^{3+}). Our results (Fig. 4a) indicate that enterochelin synthesis is less readily induced by conditions of iron stress than the aerobactin system, since significant β-galactosidase activity was present in cultures (up to 10 μM dipyridyl) which showed basal levels of enterochelin. Only at dipyridyl concentrations above 50 μM was there also significant synthesis of enterochelin. Note that the bacterial strain used in this experiment (LG1689) could not synthesize aerobactin due to insertional inactivation by the Mu phage, so that all iron actively entering the cells was transported by enterochelin. Nevertheless it is clear that, except under conditions of extreme iron stress, expression of the enterochelin system at only a fraction of its maximal level was adequate to support bacterial growth in defined medium.

Thus, in addition to its greater effectiveness at low concentrations, and to the more efficient, directed use of the iron it sequesters, aerobactin has the advantage that its synthesis is induced more readily in response to conditions of iron-limitation. It has been suggested that iron-uptake systems in enteric bacteria have common regulatory components. For example, mutations in the fur (ferric uptake regulation) locus cause constitutive expression of both the enterochelin and aerobactin systems (6,7,17). Using indicator plates, we have isolated spontaneous mutants of strain LG1689 which show high levels of β-galactosidase synthesis even in the presence of high concentrations of iron (Fig. 4b). Mutants such

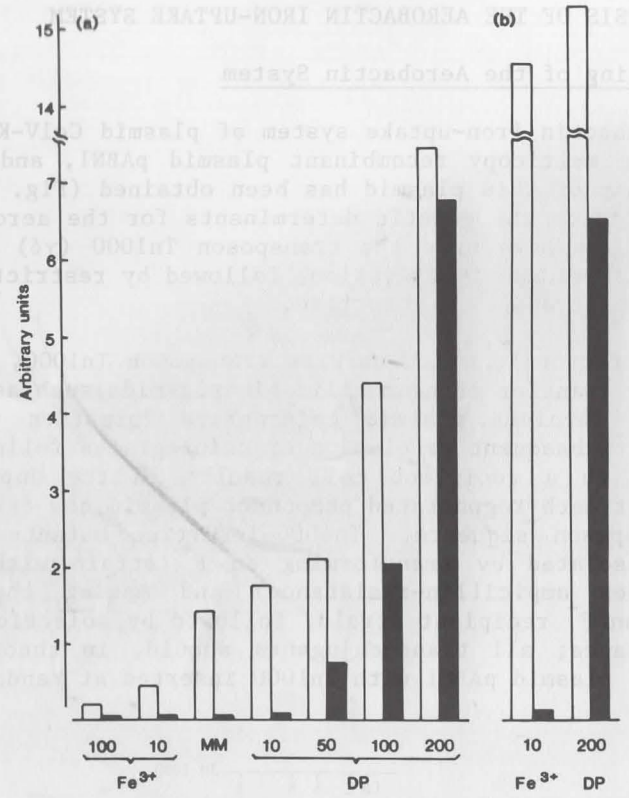

Fig. 4. Effect of iron stress on expression of the aerobactin and enterochelin iron-uptake systems. Strains LG1689 (a) and LG1712 (b) were grown overnight in M9 medium (MM) containing Fe^{3+} (as $FeCl_3$) or α,α'-dipyridyl (DP) at the concentrations indicated (μM). Cultures were diluted in the same media to an optical density at 450 nm of 0.1, incubated with aeration at 37°C for 2 hr and assayed for enterochelin and β-galactosidase. Results are displayed in arbitrary units in order to compare directly the range of detectable levels of enterochelin (approximately 1 to 50 μM) with β-galactosidase activities up to approximately 200 Units.

as LG1712 carry chromosomal mutations causing constitutive expression of the system which modulates aerobactin biosynthesis, but unlike fur mutants they showed apparently normal regulation of the enterochelin genes. These observations, and our data showing that the 2 systems have markedly different sensitivities to iron stress, strongly suggest the action of rather more complex regulatory mechanisms than have been proposed (6,7,17).

GENETIC ANALYSIS OF THE AEROBACTIN IRON-UPTAKE SYSTEM

Physical Mapping of the Aerobactin System

The aerobactin iron-uptake system of plasmid ColV-K30 has been cloned as the multicopy recombinant plasmid pABN1, and an initial restriction map of this plasmid has been obtained (Fig. 5, Ref. 3). In order to locate the genetic determinants for the aerobactin system on pABN1, we have used the transposon Tn1000 ($\gamma\delta$) to generate mutants by insertional inactivation, followed by restriction mapping of the sites of transposon insertion.

The sex factor F, which carries transposon Tn1000, can mediate low frequency transfer of nonmobilizable plasmids such as pABN1 by a process that involves plasmid cointegrate formation promoted by Tn1000 (16). Subsequent resolution of cointegrates following conjugal transfer to a recipient cell results in the duplication of Tn1000 so that each regenerated component plasmid now carries a copy of the transposon sequence. Tn1000-insertion mutants of plasmid pABN1 were isolated by transforming an F^+ strain with pABN1 DNA (which encodes ampicillin-resistance) and mating the resultant strain with an F^- recipient strain, followed by selection for ampicillin-resistance; all transconjugants should, in theory, carry a derivative of plasmid pABN1 with Tn1000 inserted at random.

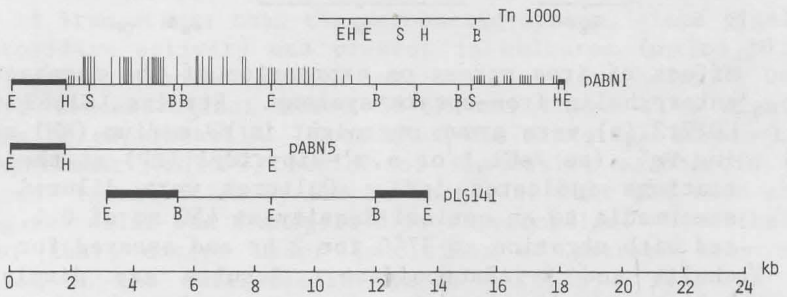

Fig. 5. Physical and genetic maps of pABN1 and of derived subclones. Restriction sites for the enzymes EcoR1 (E), HindIII (H), BamH1 (B), and SalI (S) are indicated for pABN1 and Tn1000. Long stalks on pABN1 indicate insertions of Tn1000 inactivating aerobactin production, while the intermediate-length stalks represent insertions which result in cloacin-resistance (i.e., which inactivate receptor activity). Short stalks indicate insertions which leave the whole iron-uptake system intact. Plasmids pABN5 and pLG141 are subclones of pABN1; the vector (represented by thick lines) in plasmids pABN1 and pABN5 is pPlac (3), and that in plasmid pLG141 is pACYC184.

Transconjugants were screened for aerobactin production using an indicator strain designated LG1522; this has the iuc mutant plasmid from LG1418 (specifying normal aerobactin uptake) in a fepA genetic background (in which ferric-enterochelin transport is defective), and will therefore grow on iron-deficient medium only when supplied with exogenous aerobactin. Of 450 strains assayed for their ability to cross-feed LG1522, 220 (49%) were found to be unable to secrete siderophore, and the transposon insertion site in 30 of these was determined by restriction analysis of plasmid DNA. The sites of insertion in these aerobactin-deficient mutant plasmids (indicated by the long stalks in Fig. 5) define a region of approximately 5.5 kb of the ColV-K30-derived HindIII fragment of pABN1. This localization based on transposon mutagenesis is consistent with the observation that strains carrying plasmid pABN5, a derivative comprising only the 8.6 kb EcoRI fragment of pABN1 (Fig. 5), produce aerobactin (as determined in the biological assay with strain LG1522) and so clearly contain all genes required for its biosynthesis.

Since the aerobactin receptor is also the receptor for the En. cloacae bacteriocin cloacin DF13 (2,35), sensitivity to cloacin was used to assay receptor synthesis among strains carrying pABN1::-Tn1000 plasmids. Of 70 aerobactin-producing strains assayed in this way, 11 (16%) were cloacin DF13-resistant, and the sites of Tn1000 insertion in the plasmids of these and of 35 cloacin-sensitive strains were determined by restriction analysis. In cloacin-resistant strains, insertion sites (indicated by the intermediate-length stalks in Fig. 5) spanned a region of approximately 2 kb which includes the central EcoRI site of pABN1. This is consistent with the observation that neither EcoRI fragment of pABN1, when cloned alone, conferred cloacin DF13-sensitivity on the host cell (3). Furthermore, the defined region is within the 6.5 kb BamHI fragment of pABN1, which, when cloned into a vector plasmid, was shown to specify receptor activity as indicated by sensitivity to cloacin DF13 (21).

Expression of pABN1 and Derivative Plasmids in Maxicells and Minicells

In order to determine the polypeptide products of the aerobactin system, pABN1 and derivative plasmids were expressed in maxicells (30) and minicells (28). The cloned fragment in pABN1 specified the synthesis of 5 polypeptides with apparent molecular weights of 74,000; 62,000; 50,000; 45,000; and 35,000 in both the minicell (Fig. 6) and maxicell (data not shown) expression systems. All except the 74,000 d polypeptide were also produced by pABN5; however, pABN5 encodes an additional polypeptide of 34,000 d which may be a truncated form of the 74,000 d product. The 6.5 kb BamHI fragment of pABN1, which specifies receptor activity when cloned in vector

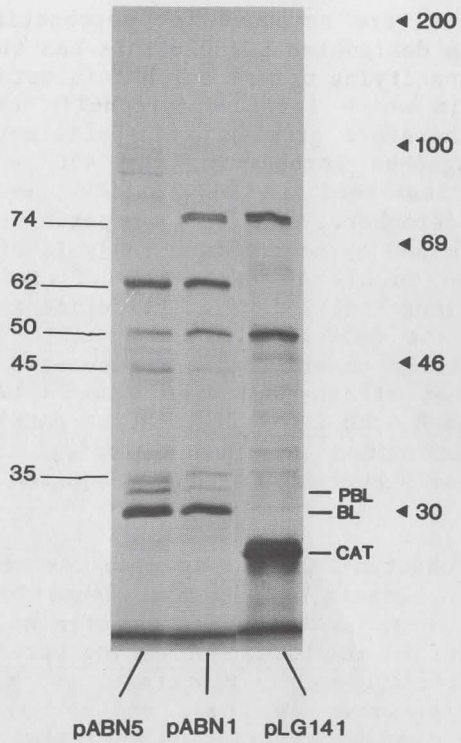

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of polypeptides expressed in minicells harboring plasmid pABN1 and derivatives. Mobilities of standard molecular weight markers are indicated (in kilodaltons) on the right-hand side. Translation products of pABN5, pABN1, and pLG141 are shown with an indication of their molecular weights; the 74,000 dalton polypeptide is visible in its mature and precursor forms. BL and PBL indicate pPlac-encoded β-lactamase and pre-β-lactamase, respectively, and CAT is pACYC184-encoded chloramphenicol acetyl transferase.

plasmid pACYC184 (as in pLG141), encodes 2 polypeptides (M_r = 74,000 and 50,000).

Two approaches were used to determine the linear order of genes specifying the polypeptides required for aerobactin biosynthesis. The first involved expression of selected aerobactin-deficient pABN1::Tn1000 mutant plasmids in maxicells. The second approach was to construct derivatives of selected mutants to give a series of plasmids containing varying lengths of the siderophore biosynthesis region of pABN1. These derivative plasmids were expressed in mini-

cells to determine their polypeptide products. Taken together, results from the 2 methods indicated a linear map of the translation products of plasmid pABN1 as shown in Fig. 7. The presence in some gels of putative truncated forms of the 74,000; 62,000; and 50,000 dalton polypeptides suggests that the direction of transcription, at least for 3 of the genes, is from left to right of the map, as drawn (Fig. 7).

Interestingly, insertions of the 5.7 kb transposon Tn1000 did not necessarily marked any polar effects. For example, mutants deficient in siderophore biosynthesis were all cloacin-sensitive, indicating some receptor expression, and moreover, expression of these mutants in maxicells and minicells generally showed the loss of only a single polypeptide. These data may indicate that the system is not expressed as a single operon, but instead has several promoter sites, possibly 1 for each gene. However, insertions of Tn1000 into a small region at the proximal end of the gene encoding the 62,000 d polypeptide did show polar effects, markedly reducing synthesis of all 5 polypeptides in the system. It is possible that these insertions identify an essential control region for the aerobactin system; the nature and possible role of this region are currently under investigation in our laboratory.

The Biosynthesis of Aerobactin

We have shown that the aerobactin system of plasmid ColV-K30 specifies 5 polypeptides, 4 of which are involved in aerobactin biosynthesis, the fifth being the 74,000 d outer-membrane protein that serves as the receptor for ferric-aerobactin. The aerobactin molecule comprises 2 N-acetylated, N-hydroxylated lysine derivatives linked to the α and γ carboxyl groups of a single residue of citric acid (Fig. 8, Ref. 14). Although the specific pathway of synthesis from existing lysine and citric acid has not yet been characterized, it is not unreasonable to speculate, in the light of our evidence, that: 4 genes are required for this synthesis, 2 specific enzymes mediate acetylation and hydroxylation of lysine, and 2 more effect condensation of these derivatives to the citric acid moiety. It is hoped that a greater understanding of the precise biochemical roles of the 4 polypeptides we have identified will allow the development of inhibitors or analogs which may be of therapeutic value in the control of invasive bacterial infections.

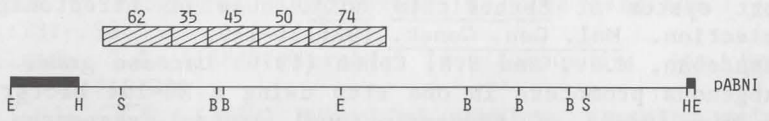

Fig. 7. Translational map of the iron-uptake region of plasmid pABN1. Numbers above the shaded boxes denote polypeptide sizes in kilodaltons.

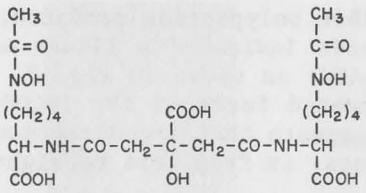

Fig. 8. The structure of aerobactin.

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PLASMID-MEDIATED IRON SEQUESTERING SYSTEMS IN PATHOGENIC STRAINS OF VIBRIO ANGUILLARUM AND ESCHERICHIA COLI

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INTRODUCTION

Properties of the host vertebrate and invading bacteria define the conditions that precipitate the onset of an infectious process. In disseminated infections, an important bacterial attribute is the ability to grow in the host vertebrate's body fluids and tissues.

An essential element for bacterial growth is iron, which is found as a component of many bacterial cellular processes (29). However, in vertebrate fluids, iron is bound by high affinity iron-binding proteins and thus the concentration of available iron is far too low for bacterial utilization (6). Certain pathogenic isolates of Vibrio anguillarum and Escherichia coli have developed very efficient plasmid-mediated iron-sequestering mechanisms (7,8,31). These systems encoded by pJM1 in V. anguillarum and pColV-K30 in E. coli, responsible for the high-virulence phenotype of these bacteria, have been genetically characterized (9,11,12,28,32). The analysis of mutants in these 2 systems led to the demonstration of the existence of 2 components: a siderophore and a receptor for the iron-siderophore complexes. In the case of pColV-K30 the siderophore was identified as aerobactin (30), a hydroxamate compound originally found in culture supernatants of Aerobacter aerogenes (14). The receptor was determined to be a 74 kilodalton (kd) outer membrane protein (4,15). Recently, the genetic determinants for aerobactin and its receptor have been cloned (3).

In the case of V. anguillarum, concomitant with efficient plasmid-mediated iron uptake, there was an induction of the synthe-

sis of 2 novel outer membrane proteins. One of them, the 86 kd OM2 protein was associated with the presence of pJM1 (10). Analysis of transposition-generated mutants suggested that OM2 is a receptor for complexes of iron with a siderophore produced by V. anguillarum cells harboring an intact pJM1 plasmid (28).

The present work describes the molecular cloning and expression of genetic determinants of the pJM1 plasmid-mediated iron-uptake system of V. anguillarum. A component of this system, the iron-regulated outer-membrane protein OM2, is encoded by the pJM1 plasmid and may play a role as a receptor for complexes of iron with the V. anguillarum siderophore anguibactin.

It is also reported here that although the pJM1 and pColV-K30 plasmid-mediated iron uptake systems are phylogenetically unrelated (27), they share a common feature: the iron uptake regions in both plasmids are flanked by repeated sequences. These characterisitcs may have played a role in the epidemiological spread of these virulence factors.

RESULTS AND DISCUSSION

Cloning of pJM1 DNA Genes Involved in the Iron Uptake Process.

The pJM1 DNA fragments obtained by partial digestion with the restriction endonuclease XhoI were ligated with the vector pVK102 (17) cleaved with the same enzyme. The ligated DNA was in vitro packaged and the phage particles were used to transduce E. coli HB101. Based upon previous knowledge of the iron-uptake region in pJM1 (28), clones carrying DNA fragments associated with the iron-uptake sequences were selected for further studies. The pJM1 DNA fragments contained in the clones pJHC-T2, pJHC-T7, pJHC-T11, and pJHC-T2612 are shown in Fig. 1. Each of these clones was conjugated into different iron uptake-deficient low-virulence V. anguillarum strains as previously described (24) by using the helper plasmid pRK2013 (13). The V. anguillarum recipients were the plasmidless H775-3, the 775::Tn1-5 strain harboring the Tn1 insertion derivative pJHC-91 and 775::Tn1-6 carrying the deletion plasmid pJHC9-8 (Fig. 1). The plasmids carried by each V. anguillarum exconjugant are listed in Tab. 1. Analysis of the production of siderophore activity was carried out by testing culture supernatants in bioassays using as indicator strain V. anguillarum 775:Tn1-5 (28). The results shown in Tab. 2 indicated that exconjugants harboring either the cosmid pJHC-T7 or pJHC-T2612, in addition to an indigenous pJM1 derivative such as pJHC-91 or the deleted plasmid pJHC9-8 exhibited a level of siderophore activity comparable to that produced by the wild-type V. anguillarum 775. Exconjugants carrying only the clone pJHC-T7 or pJHC-T2612 produced a very low level of siderophore activity. No siderophore activity was detected in V. anguillarum harboring either the control pVK102, the clones pJHC-T2, pJHC-T11, or the indigenous plasmids pJHC-91 or pJHC9-8. The present data suggest that an additional factor present in the indigenous plasmids and not in the recombinant clones is necessary for a production of a normal level of siderophore activity (24). The possibility of this factor to be involved in the export of siderophore outside the cell was ruled out by performing bioassays with total homogenates of the cells (24). Investigation of the production of the pJM1-encoded receptor, using each of the exconjugants strains as indicator in bioassays showed that 3 cosmid clones pJHC-T7, pJHC-T11, and pJHC-T2612 carry information for production of receptor activity while neither the clone pJHC-T2 nor the control plasmid pVK102 did (Tab. 2). Concomitantly with the presence of receptor activity, induction of the outer membrane protein OM2 was found under iron-limiting conditions. Since the ability to induce siderophore and receptor acti-

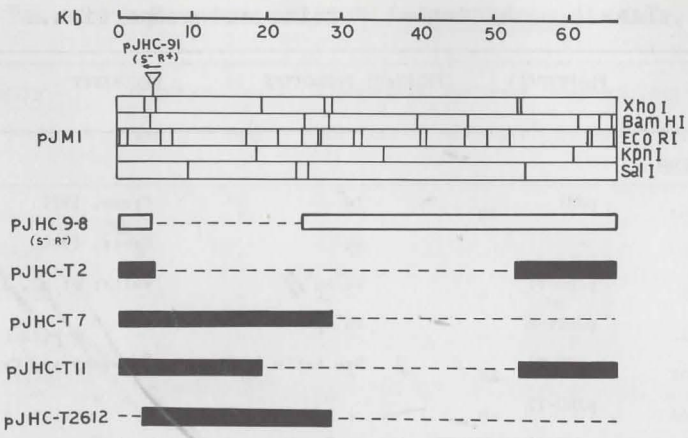


Fig. 1. Physical map of pJM1 derivatives and recombinant clones. The symbol ▽ indicates the site of the Tn1 insertion in the pJM1 derivative pJHC-91 while the arrow over this symbol gives the orientation of the Tn1 transposition sequence with respect to β-lactamase gene transcription. Also noted in this figure is the phenotype of the V. anguillarum strain carrying the pJHC-91 plasmid. Iron uptake-deficient derivatives are classified depending on whether the Tn1 insertion or deletion affected the genes associated with the production or activity of either siderophore (S^-R^+) receptor (S^+R^-). Dashed lines correspond to pJM1 DNA regions that were either deleted (i.e., in pJHC9-8) or that were not included in the cloned DNA. Thicker lines represent the pJM1 DNA cloned in the cosmid vector pVK102 (17). The clone pJHC-T2, pJHC-T7, pJHC-T11, and pJHC-T2612 were obtained by cloning XhoI partial digests of pJM1 DNA.

Tab. 1. Bacterial strains and properties.

STRAIN	PLASMID(S)	RELEVANT PHENOTYPE	REFERENCE
V. anguillarum			
775	pJM1	Iu^+	Crosa, 1977
H775-3	-	Iu^-	Crosa, 1980
775::Tn*1*-5	pJHC-91	Iu^-Ap^r	Walter et al, 1983
775::Tn*1*-6	pJHC9-8	Iu^-Ap^r	"
H775-3A	pJHC-T7	See table 2	Tolmasky and Crosa, 1984
H775-3D	pJHC-T2	"	"
775::Tn*1*-5A	pJHC-91, pJHC-T7	"	"
775::Tn*1*-6A	pJHC9-8, pJHC-T7	"	"
775::Tn*1*-5B	pJHC-91, pJHC-T2612	"	"
775::Tn*1*-6B	pJHC9-8, pJHC-T2612	"	"
H775-3B	pJHC-T2612	"	"
H775-3C	pJHC-T2	"	"
5822	-	Iu^+	This laboratory
E. coli			
HB101	-	*pro*, *leu*, *thi*, *lacY*, *hsr*$^-$, *hsm*$^-$, *endoI*$^-$, *recA*	Boyer and Roulland-Dussoix,1969
C600	-	*thi*, *thr*, *leu*, *lacY* *supE44*, *tonA*, $^-$	Appleyard, 1954
CHBA187	Five criptic plasmids	aerobactin producing	Children's Hospital Buenos Aires, Argentina.

Iu: iron uptake; Ap: ampicillin.

vity should be correlated with the iron-uptake proficiency, the iron-uptake properties of the exconjugants were tested by measuring the MIC of the iron-chelating agent ethylenediamine-di(o-hydroxy-phenyl acetic acid) (EDDA). Tab. 2 shows that *V. anguillarum* harboring the indigenous plasmid pJHC-91 or pJHC9-8 increase the MIC for EDDA from 2 to 40 μM upon the introduction of either pJHC-T7 or pJHC-T2612, becoming iron-uptake proficient. Introduction of either of these clones in the plasmidless *V. anguillarum* H775-3 resulted in

Tab. 2. Properties of *V. anguillarum* strains.

STRAIN	UPTAKE OF ^{55}Fe[a] -ang	+ang	SIDEROPHORE ACTIVITY[b]	RECEPTOR ACTIVITY[c]	PRESENCE OF OM2[d]	MIC for EDDA[e]
775	+	+	+	NA	+	40
H775-3	-	-	-	-	-	2
775::Tn*1*-5	-	-	-	+	+	2
775::Tn*1*-6	-	-	-	-	-	2
775::Tn*1*-5A	ND	ND	+	NA	+	40
775::Tn*1*-6A	ND	ND	+	NA	+	40
H775-3A	-	+	low	+	+	2
775::Tn*1*-5B	ND	ND	+	NA	+	40
775::Tn*1*-6B	ND	ND	+	NA	+	40
H775-3B	ND	ND	low	+	+	40
H775-3C	ND	ND	-	+	+	2
H775-3D	-	-	-	-	-	2
H775-3(pVK102)	ND	ND	-	-	-	2

[a] ^{55}Fe uptake was determined as described in the legend to Fig. 3. ang: cell-free supernatant of a *V. anguillarum* 775 culture which contains the pJM1 plasmid mediated siderophore anguibactin.

[b] Anguibactin production was determined by testing the ability of cell-free supernatant from cultures of each strain to cross-feed the indicator strain 775::Tn*1*-5 in bioassays as described previously (Walter et al., 1983).

[c] Receptor activity was scored by using each strain as indicator in bioassays with crude preparations of anguibactin.

[d] The presence of OM2 was tested in SDS-polyacrylamide gel elctrophoresis (SDS-PAGE) of outer membrane proteins obtained from cells of each strain cultured under iron-limiting conditions as previously described (Crosa and Hodges, 1981).

[e] Minimal inhibitory concentration (MIC) for ethylenediamine-di(o-hydroxyphenyl acetic acid) (EDDA) was determined on M9 minimal medium agarose plates containing increasing concentrations of EDDA. ND: not determined; NA: not applicable.

no modification of their MIC for EDDA, correlating with their low siderophore activity. It can also be seen in Tab. 2 that neither pJHC-T11 nor pJHC-T2 nor the control pVK102 change the iron-uptake deficient phenotype of the V. anguillarum recipients. Since these exconjugants have an active receptor, the low amount of siderophore activity must be responsible for the iron uptake-deficient phenotype. Thus, the additional factor mediated by the indigenous plasmids pJHC-91 and pJHC9-8, which is involved in the production of an increased siderophore activity, may be necessary for the ability to grow under iron-limiting conditions. This transacting factor could play a role on the regulation of the expression of the siderophore genes. Since the enhancing effect also occurs in strains harboring pJHC9-8, in which the iron-uptake region is deleted, the transacting regulatory genes must be encoded on pJM1 sequences other than the iron-uptake regions as defined by Tn1 transposition mutagenesis.

The exconjugant V. anguillarum strain 775:Tn1-5A which harbors the plasmids pJHC-91 and pJHC-T7 was also tested in experimental infections. The results showed that this strain possesses a high-virulence phenotype as compared to the original strain V. anguillarum 775::Tn1-5 which only carries pJHC-91 and was no virulent. This result demonstrated the intimate relation existing between the iron-uptake phenotype and the virulence characteristics of V. anguillarum.

Role and Origin of the OM2 Protein

The outer-membrane protein OM2 has been tentatively postulated as the receptor for the complex iron-siderophore in V. anguillarum (24,28). The OM2 protein was induced under iron-limiting conditions only if the cells harbored the pJM1 plasmid, suggesting that it was plasmid-encoded or that it had a chromosomal origin but was induced in response to a plasmid-specific substance. To determine whether the OM2 structural genes were present in pJM1, recombinant clones containing restriction fragments with the iron uptake-region sequences were introduced in E. coli HB101 cells to rule out the contribution of the V. anguillarum chromosome (1). Immunoblot analysis of either total cell lysates or outer membranes from those cells was used to detect the presence of OM2. The results, shown in Fig. 2, indicated that OM2 was present in both the total cell lysates as well as in the outer membranes of E. coli harboring clones containing the pJM1 BamHI fragment 1 (pJHC-W1), the EcoRI fragment 2 (pJHC-P100), or the SalI fragment 3 (pJHC-P102) (see physical map, Fig. 1). E. coli cells harboring clones containing other pJM1 fragments (pJHC-P101) as well as plasmidless derivatives did not synthesize the OM2 protein. Thus, these results showed that the structural genes for the OM2 protein must be present in the plasmid pJM1.

To determine the contribution of OM2 as a receptor in the iron-transport process, the kinetics of radioactive iron uptake was

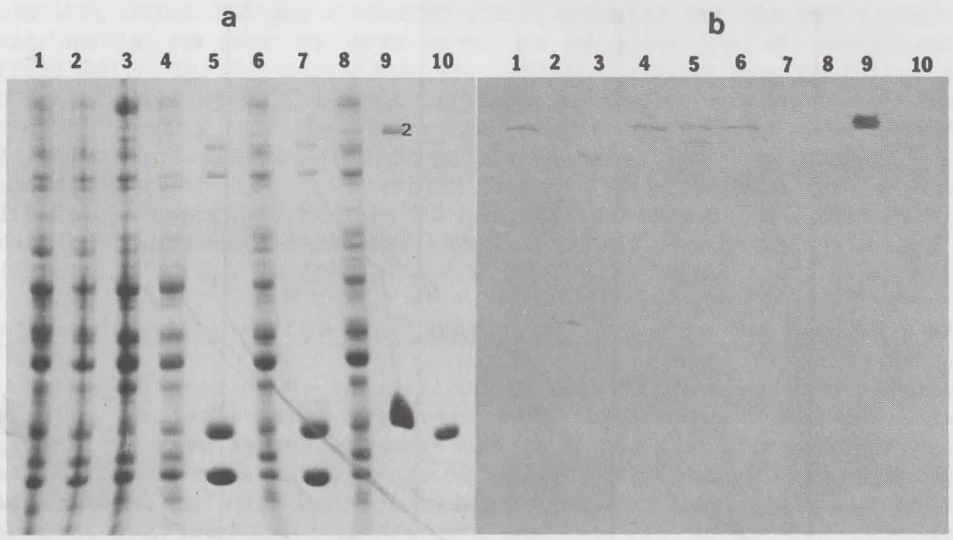


Fig. 2. SDS-PAGE and immunoblot analysis of total and outer membranes. Panel a: SDS-PAGE stained with Coomasie blue of total cell lysates (lanes 1-4, 6, and 8) and outer membranes proteins (lanes 5,7,9, and 10) of V. anguillarum cells (lanes 9 and 10) and E. coli HB101 cells (lanes 1-6) harboring recombinant plasmids containing different pJM1 fragments. Plasmidless E. coli HB101 cells were used as negative control (lanes 7 and 8). The pJM1 fragments cloned in HB101 cells were lane 1, SalI fragment 3 (pJHC-P102); lane 2, SalI fragment 2 (pJHC-P101); lane 3, EcoR1 fragment 7 (pJHC-Y52); lane 4, EcoR1 fragment 2 (pJHC-P101); and lanes 5 and 6, BamHI fragment 1 (pJHC-W1). Panel b: immunoblot of the gel in panel a stained with horse-radish peroxidase conjugated Protein A H_2O_2 and HRP color developing reagent (Bio-Rad, Richmond, California). The number 2 in panel a, lane 9 identifies the outer membrane protein OM2.

examined for V. anguillarum strains harboring indigenous and recombinant plasmids. V. anguillarum 775::Tn1-5 which harbors pJHC-91 and produces OM2 but not the siderophore anguibactin, was unable to take up iron under conditions in which the wild-type 775 did. However, when cell-free supernatant from V. anguillarum 775 which contains anguibactin was added in conjunction with ^{55}Fe, the iron-uptake kinetics was similar to that of V. anguillarum 775 (Fig. 3b). V. anguillarum cells carrying the recombinant cosmid pJHC-T7, which encodes the OM2 protein and receptor activity, were able to take up iron only in the presence of cell-free supernatant from a culture of V. anguillarum 775 (Fig. 3c). Conversely, V. anguillarum carrying

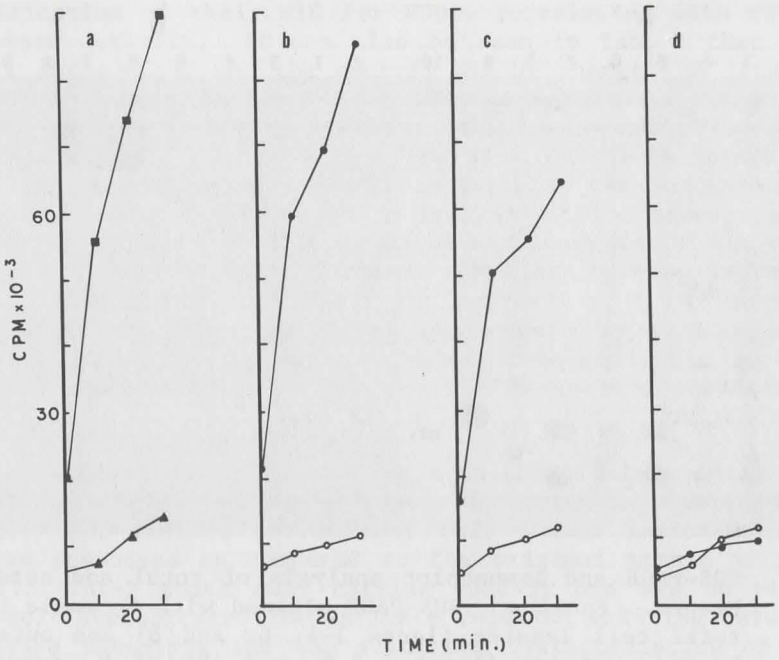


Fig. 3. Kinetics of radioactive ^{55}Fe iron uptake by nongrowing cells of various V. anguillarum strains under iron-limiting conditions. V. anguillarum strains were grown for several generations at room temperature (24°C) in M9 minimal medium containing the iron chelator EDDA at 10 μM (for iron uptake-proficient bacteria) or 2 μM EDDA (for iron uptake-deficient bacteria). Exponentially growing cultures were centrifuged and cells were washed and resuspended in the same volume of similar medium containing the required aminoacids aspartic acid and histidine at 20 μg/ml. After further incubation for 2 hours at room temperature to deplete iron and aminoacid intracellular pools, cultures were cetrifuged and cells were washed and resuspended to an absorbance value of 1.00 at 590 nm, either in a similar medium lacking essential aminoacids but containing 100 μM sodium nitrilotriacetate or cell-free supernatant containing siderophore activity from V. anguillarum 775(pJM1) grown in the same medium. The iron-uptake kinetics was performed using 1 μCi/ml of carrier-free $^{55}FeCl_3$, as described previously (10). Panel a, strains 775(pJM1) (■) and H775-3 (▲); panel b, strain 775::Tn1-5(pJHC-91); panel c, strain H775-3A(pJHC-T7); panel d, strain H775-3D(pJHC-T2). In panels b, c, and d the experiments were carried out either in the presence (●) or absence (o) of cell-free supernatants from V. anguillarum 775(pJM1).

pJHC-T2, which did not encode receptor activity nor the OM2 protein, was unable to take up iron even in the presence of anguibactin (Fig. 2d). In all cases the iron-uptake process was inhibited by KCN (data not shown), suggesting that it must be uptake rather than simple binding to the bacterial membranes. Thus, the energy-dependent plasmid-mediated iron transport in V. anguillarum is a consequence of the interaction between anguibactin and a non-diffusible component present in the cell envelope, acting as receptor for the iron-siderophore complexes, and which may be the OM2 protein.

Repeated Sequences Flank the Iron-Uptake Region in the Plasmid pJM1

The ability of V. anguillarum to acquire iron under conditions of iron limitation was correlated with the presence of a pJM1-like plasmid in most instances, although in a few cases it was not possible to demonstrate the presence of such a plasmid (23). Nonetheless, these strains were of a high virulence phenotype and had a very active iron-sequestering system. It was speculated that the iron-uptake system present in those strains may be of a chromosomal nature and possibly related to the pJM1 plasmid-mediated iron-uptake system. Recently, Southern blot hybridization experiments indicated that pJM1 sequences were indeed present in the chromosomal DNA of these strains (20). The results suggested that the homology was, in part, due to the presence of repeated sequences in the pJM1 plasmid, that were also present in the chromosome of these strains. A detailed analysis of the pJM1 genome revealed the presence of 2 types of repeated sequences, designated RSv1 and RSv2. Two of the RSv1 sequences were found to flank the iron-uptake region of pJM1 (Fig. 4, panel a). Hybridization experiments, using a cloned pJM1 EcoR1 fragment containing the RSv1 sequences as a radiolabeled probe (plasmid pJHC-Y52), indicate that the RSv1 repeated sequence is also present in the chromosome of the plasmidless iron uptake-proficient strain 5822 (Fig. 4, panel b lanes C and D) and in the chromosome of the plasmidless iron uptake-deficient H775-3 strain (lanes A and B). The RSv2 sequences were only detected in the chromosomal DNA of the plasmidless iron uptake-deficient H775-3 strain (data not shown). The RSv1 sequences flanked the pJM1 iron-uptake region and were also present in the 5822 chromosome. Therefore, it was of interest to determine whether the pJM1 iron-uptake region was also included in the chromosome of this strain. Consequently, hybridization experiments were carried out between 5822 chromosomal DNA and the radiolabeled plasmid pJHC-P100 which contained a 9.6-kb EcoRI fragment internal to the iron-uptake region (Fig. 4, panel a). The results indicate that chromosomal DNA from strain 5822 shows homology with this probe (Fig. 4, panel B, lanes I and J), while the plasmidless iron uptake-deficient strain H775-3 did not (lanes G and H), although this strain hybridized with the RSv1 probe.

Thus, not only pJM1 iron-uptake sequences but also RSv1 sequences which flank the iron-uptake region in pJM1, occur in the

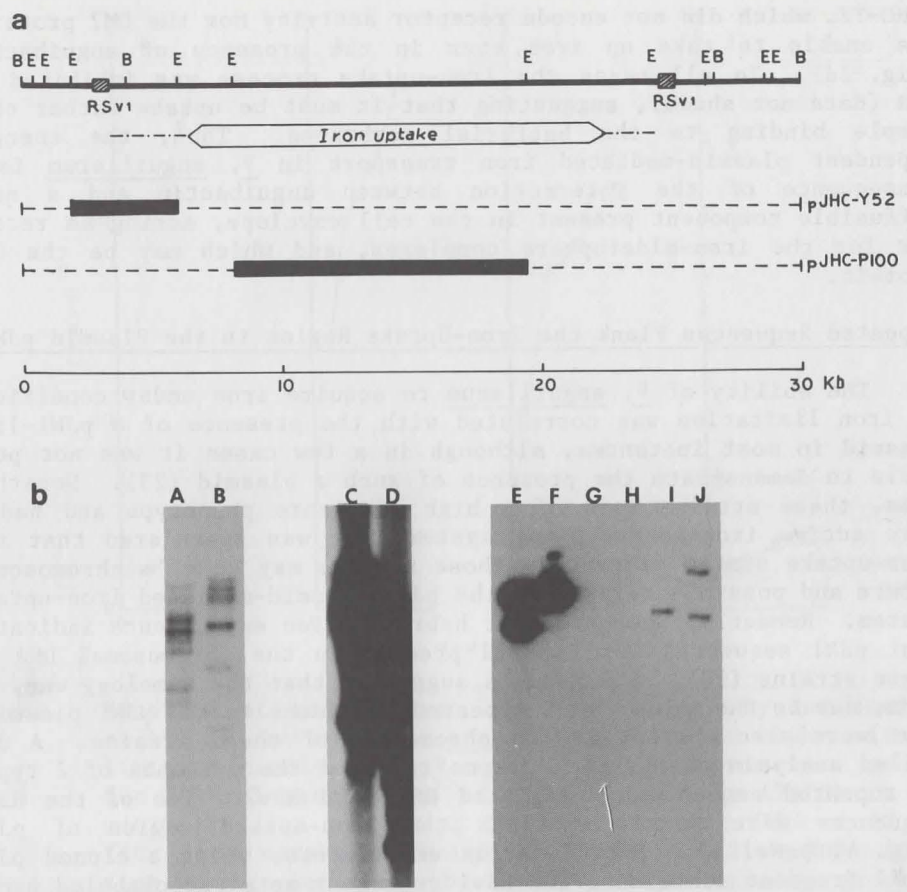
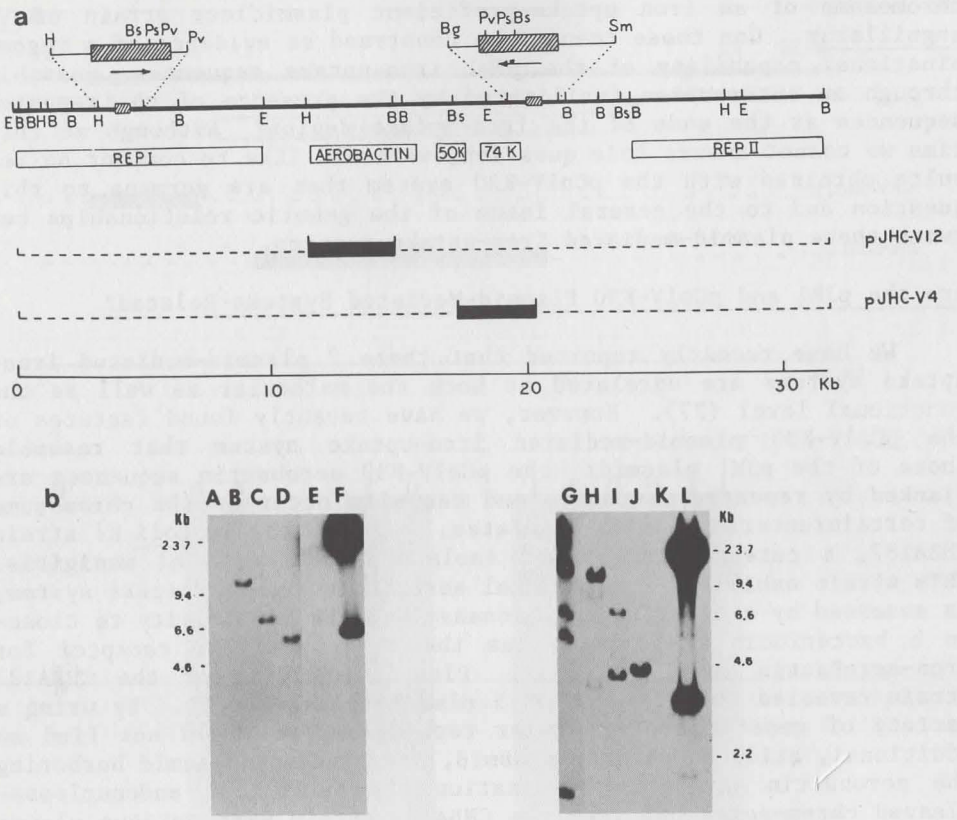


Fig. 4. Panel a: Physical map of the iron-uptake region in pJm1. The position of 2 copies of the repeated sequences RSv1 is indicated by hatched boxes. Thicker lines represent cloned EcoRI cleaved pJM1 DNA fragments. EcoRI fragment 7 cloned in pKY2700 (pJHC-Y52) contains one copy of the repeated sequence RSv1. EcoRI fragment 2 cloned in pBR325 (pJHC-P100) is internal to the iron-uptake region. Dashed lines correspond to pJM1 DNA regions that were not included in the cloned DNA. Panel b: Southern blot hybridization using the clones pJHC-Y52 and pJHC-P100 as radioactive probes. Hybridization experiments using either pJHC-Y52 (Channels A-D), or pJHC-P100 (channels E-J), as a radioactive probe against V. anguillarum chromosomal DNA (A-D and G-J). (A): EcoRI cleaved 775-3; (B): BamHI cleaved 775-3; (C): EcoRI cleaved 5822; (D): BamHI cleaved 5822 DNA1; (G): EcoRI cleaved 775-3; (H): BamHI cleaved 775-3; (I): EcoRI cleaved 5822 and (J): BamHI cleaved 5822. Lanes E and F show EcoRI cleaved DNA of plasmids pJHC-P100 (lane E) and pJM1 (lane F).

chromosome of an iron uptake-proficient plasmidless strain of V. anguillarum. Can these results be construed as evidence of a recombinational capability of the pJM1 iron-uptake sequences, possibly through an interaction facilitated by the presence of the repeated sequences at the ends of the iron-uptake region? Although at this time we cannot answer this question, we would like to comment on results obtained with the pColV-K30 system that are germane to this question and to the general issue of the genetic relationships between these plasmid-mediated iron-uptake systems.

Are the pJM1 and pColV-K30 Plasmid-Mediated Systems Related?

We have recently reported that these 2 plasmid-mediated iron-uptake systems are unrelated at both the molecular as well as the functional level (27). However, we have recently found features of the pColV-K30 plasmid-mediated iron-uptake system that resemble those of the pJM1 plasmid: the pColV-K30 aerobactin sequences are flanked by repeated sequences and can also occur in the chromosome of certain enteric clinical isolates, such as, the E. coli K1 strain CHBA187, a cerebrospinal fluid isolate from a case of menigitis. This strain exhibited a functional aerobactin iron-transport system, as assessed by a crossfeeding bioassay and its sensitivity to cloacin a bacteriocin that recognizes the outer membrane receptor for iron-aerobactin complexes (26). Plasmid analysis of the CHBA187 strain revealed the presence of 5 plasmid classes (25). By using a variety of genetic and molecular techniques, we could not find an additional, still undetected plasmid, nor find any plasmid harboring the aerobactin genes. Hybridization of restriction endonuclease-cleaved chromosomal DNA from the CHBA187 strain with various clones containing subsets of the pColV-K30 aerobactin region, such as, pJHC-V4 and pJHC-V12 (See Fig. 5, panel a), demonstrated that the genetic determinants for the aerobactin iron-uptake system were located on a 10.5 kb HindIII fragment of the CHBA187 chromosome (Fig. 5, lanes B and H). Therefore, the aerobactin region in the chromosome is part of a HindIII restriction endounclease fragment about 6 kb smaller than the HindIII fragment in which they are found in the pColV-K30 plasmid (3; Fig. 5 panel a). However, the chromosomal aerobactin and receptor regions appeared to be highly conserved, as compared to the homologous regions in the plasmid pColV-K30. Thus, Fig. 5, panel b, lanes C and D, show that the distances HindIII-EcoRI and HindIII-BstEII mapped along the left end of the 16.3 kb pColV-K30 HindIII fragment (Fig. 5, panel a) were identical to those found in the CHBA187 aerobactin region as determined by successive digestions with various restriction endonucleases followed by hybridization with the aforementioned probes. The fact that the BstEII fragment, which in pColV-K30 contains the end of the receptor genes and part of the flanking sequences (Fig. 5, panel a), possessed a larger size in the case of the chromosomal BstEII digest (4 kb as compared to 3 kb in pColV-K30; see Fig. 5, panel b, lanes J and L) indicated that considerable rearrangement occurred in the



right-flanking area of the aerobactin chromosomal region. Furthermore, the recombinant plasmid pJHC-V4 containing the right-flanking sequence hybridized not only with the 10.5-kb HindIII chromosomal fragment but also with 2 other fragments, suggesting that sequences contained in this probe were repeated in the CHBA187 chromosome (Fig. 5, panel b, lanes H and I). These sequences were also found in the chromosome of the E. coli C600 strain, although its DNA did not show any hybridization with the pJHC-V12 plasmid, a specific probe for the aerobactin region (Fig. 5, panels a and b, lanes A and G), nor did the strain produce aerobactin or possess a cloacin-sensitive phenotype.

These results prompted us to study the sequences flanking the iron-uptake region in the pColV-K30 plasmid since their knowledge could shed light in understanding the high degree of conservation and ubiquity of these genes. Therefore, hybridization and restriction mapping experiments were carried out to determine and characterize the nature of these flanking sequences. The results indicated that repeated sequences of about 800 bp flanked the aerobactin iron-uptake region (21; Fig. 5, panel a). Restriction endonuclease analysis and hybridization with probes containing known insertion elements demonstrated that the repeated sequences were homologous to IS1 elements (21,29). Similar findings were recently reported (18,19).

The IS1-like sequence occurring at the left end of the aerobactin region, was in reverse orientation as compared to the IS1-like sequence found at the right end of this region (21; Fig. 5, panel a).

Fig. 5. Panel a: Diagram showing the location of replications regions region. The top solid line represents a restriction endonuclease map of a pColV-K30 DNA fragment containing both the aerobactin and IS1-like sequences which are both flanking the pColV-K30 aerobactin region. The restriction endonuclease sites are: E: EcoRI, B: BamHl, H: HindIII, Bs: BstE II, Ps: PstI, Pv: PvuII, Bg: BglII, Sm: SmaI. BglII, PvuII and SmaI restriction sites are not completely mapped. Hatched boxes indicate the position of the IS1-like sequences. In an amplified scale on top are shown the restriction sites within the IS1-like elements. Arrows indicate the orientation of IS1-like elements with respect to each other. Open boxes indicate DNA regions corresponding to the aerobactin siderophore, 50 K and 74 K proteins, and also the replication regions REP I and REP II adjacent to the aerobactin region. K indicates kilodaltons. REP I region is located on a 10-kb EcoRI fragment whereas the REP II region is located on a 7.6-kb BamHI fragment. Thicker lines represent 2 cloned pColV-K30 DNA fragments. The pJHC-V12 is a recombinant plasmid containing a 3.4-kb HindIII-BamHi fragment which includes part of the aerobactin genes cloned in pVK101 (17). The pJHC-V4 contains a 3-kb BstEII fragment cloned in pJBK45, (16). Panel b: Southern blot hybridization of restriction endonuclease cleaved-chromosomal DNA from strain CHBA187 with probes containing different portions of the aerobactin region. Lanes A and G: C600 DNA; lanes B-D and H-J: CHBA187 DNA; lanes E and K: lambda DNA, lanes F and L: pColV-K30 16.3-kb HindIII DNA fragment containing the aerobactin region. The restriction endonucleases used were: Lanes A, B, E, G, H, and K: HindIII; lanes C and I: EcoRI and HindIII successive digestions; lanes D, F, J, and L: HindIII and BstEII successive digestions. Lanes A-L were hybridized with ^{32}P-labeled probes: lanes A-F, pJHC-12; lanes G-L, pJHC-V4.

Inverted IS1 sequences were also recently reported to flank the gene for the ST toxin, another important virulence factor that has spread to various medically important microorganisms (22). In this case the whole unit, ST-toxin genes together with the flanking IS1 elements, were shown to have transposition ability. Thus, the finding of the IS1-like elements flanking the aerobactin region in the pColV-K30 plasmid is suggestive of a potential recombinational ability because although transposition of these genes has not been demonstrated as yet, there is a potential capability for such an activity since the genes are bracketed by IS1-like elements.

Another possibility is that the presence of IS1 elements, per se, could enhance the ability of the aerobactin region to recombine by homologous recombination, via the IS1 elements, with genomes that also possess IS1 sequences. The finding of aerobactin sequences and IS1-like sequences in the same 10-kb HindIII fragment of chromosomal DNA from E. coli CHBA187 supports these hypotheses and suggests that the spread of the aerobactin sequences may be due to a high recombinational mobility (25).

As part of the genetic analysis of the pColV-K30 plasmid, its replication region was cloned by ligation of pColV-K30 DNA partially digested with EcoRI restriction endonuclease into the EcoRI site of pBR325. Since, unlike the pColV-K30 plasmid, this cloning vector requires polymerase I for its replication, we used the ability of recombinant clones to replicate in polymerase I-deficient mutants of E. coli to select, after transformation, for clones carrying the pColV-K30 replication regions. The results indicated that there were 2 replication regions in the pColV-K30 plasmid (21). Restriction endonuclease mapping and hybridization experiments located these replication regions, designated REP I and REP II, at the left and right end, respectively, of the aerobactin region (Fig. 5, panel a). The fact that the REP I and REP II replication regions flank the aerobactin system sequences may contribute to their conservation since recombinational events that conserve one of these replication regions, may also leave intact the aerobactin system, especially in deletion or insertion events in which new plasmids are generated. Thus, the combination of essential replication regions and insertion sequences adjacent to the aerobactin sequences may have played an important role in their preservation during evolution and may have contributed to their epidemiological spread in nature.

ACKNOWLEDGMENTS

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CHAIRMAN'S INTRODUCTION: SPECIALIZED FUNCTIONS

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The final session on specialized functions was concerned with studies of plasmids that determine virulence in plants and humans and others that specify antibiotic resistance in human bacterial pathogens.

Dr. R. Amasino (University of Washington, Seattle) reported the analysis of insertion mutants in the Ti plasmid of Agrobacterium tumefaciens that is responsible for the formation of tumors in a variety of plants. Many virulence (vir) genes have in this way been mapped on the plasmid in a linked cluster, and have been shown to be expressed only when the bacteria are growing in the presence of plant cells. Two of the Ti-plasmid oncogenes have been shown to code for enzymes involved in the synthesis of the plant hormone, indoleacetic acid (IAA), and have been demonstrated to have extensive functional and DNA homology with similar genes in another plant pathogenic species, the savastanoi variety of Pseudomonas syringae. Plasmids in this species, which are also capable of producing tumors on plants, were described in more detail by Dr. T. Kosuge (University of California, Davis), who also reported homology between the IAA genes of Ps. syringae and the oncogenes of the Agrobacterium Ti plasmid. Homologous genes determining IAA were also located on the chromosomal DNA in other isolates of Ps. savastanoi.

Dr. R. Clowes (University of Texas at Dallas) reported the cloning of a gene from another Pseudomonas species, aeruginosa, which determines a toxin contributing to its pathogenicity for humans. This toxin is identical to diphtheria toxin in its mode of action on mammalian cells, which are killed by the inhibition of protein synthesis when the toxin is released in the cytoplasm, but

the 2 toxins have different receptor sites. Dr. W. Goebel (Genetics and Microbiology Institute, Würzburg, West Germany) described his work on the characterization of plasmids that cause virulence in some Escherichia coli strains by the release of products that lyse red blood cells. The genes and products involved in the synthesis of such hemolysins have been characterized, and again, similar genes with identical functions have been mapped to the chromosome of other varieties of E. coli, specifically those causing acute pyelonephritis.

Finally, Dr. B. Weisblum (University of Wisconsin Medical School, Madison) reported the analysis at the molecular level of the regulation of genes conferring inducible resistance in Staphylococcus aureus to the antibiotics chloromycetin and erythromycin. Resistance to either antibiotic, which is due to the synthesis of methylating enzymes, has been shown to be regulated at the post-transcriptional level by the formation of double-stranded regions within each transcript, incorporating that part of the transcript that is required for ribosome binding, thereby interfering with the synthesis of the enzymes concerned.

CLONING OF AN ENZYMATICALLY ACTIVE SEGMENT OF THE EXOTOXIN-A GENE OF PSEUDOMONAS AERUGINOSA

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Pseudomonas aeruginosa is a common, opportunistic, nosocomial pathogen. It excretes a variety of proteins, one of which, exotoxin A, appears to be a major virulence factor (9,19,36). Exotoxin A (PE) is a 68,000 molecular weight protein (2,28) with a mechanism of action identical to that of diphtheria toxin (DT) (26). That is, it inhibits protein synthesis in mammalian cells by the transfer of the ADP-ribosyl group of NAD^+ to a specific site on the peptide, elongation factor #2 (EF2), identified in mammalian-cell protein synthesis (14,35). As in diphtheria toxin, this enzymatic activity can be expressed by only part of the toxin molecule, the A (active) fragment of 26,000 to 27,000 molecular weight that is found in stored preparations (7,35), or after chymotrypsin or elastase cleavage of the intact toxin molecule (20,30). The remaining segment of the molecule of some 41,000 molecular weight, the B (binding) fragment, like the corresponding B fragment of DT, is believed to be involved in the recognition of cell receptors and the subsequent steps of endocytosis (26). The sequence of events in the pathway of receptor-mediated endocytosis remains obscure, and its resolution might be simplified if DT or PE mutants blocked at different steps were available. Such mutants might be derived and characterized more readily if the gene were cloned, and this was the main incentive for the project described, some details of which have already been published (23). A cloned toxin gene would also be an important intermediate in the construction of hybrid toxins (25).

We chose, as the source of the PE structural gene, the high-level PE-producing strain, Ps. aeruginosa PA103 (18). The primary cloning was carried out in HB101, a restrictionless Escherichia coli

K-12 mutant (3), using a pBR322 derivative as a vector. This vector, pRO1614 (Fig. 1), was derived by Olsen et al. (24) by the insertion of a 1.85-kb PstI fragment, that carries the replication genes from the broad host-range vector, RP1, into the PstI site of pBR322 (33). Since RP1 also carries a β-lactamase gene on a Tn1 transposon, cleaved at the identical PstI site, this results in the retention of β-lactamase activity in pRO1614 as shown in Fig. 1, and permits the use of carbenicillin (Cb) as an alternative to tetracycline (Tc) in the selection of host cells carrying the plasmid.

The chromosomal DNA isolated from PA103 was partially digested with Sau3A, and following its equilibration on a 10 to 40% sucrose gradient, fractions containing fragments from 10 to 15 kb, as monitored on an agarose gel, were separated and ligated to the BamHI-cleaved pRO1614 vector. The ligated DNA was added to HB101 cells made competent by heat treatment with Ca^{++} (8,13) and samples producing 3,000 to 10,000 Cb^r colonies (approximately 3% to 9% of which were shown to be Tc-s) were spread over nitrocellulose filters that had been laid on the agar surface of the selection plates (15). Following incubation and the imprinting of replicas (37), the colonies were lysed and the released proteins fixed to the filter in situ (22). The filters were then probed using antibodies to PE (35), which were labeled by the addition of ^{125}I-protein A from Staphylococcus aureus (21). After autoradiography, clones expressing immunologically related exotoxin A molecules were readily visible (Fig. 2). Antigen-positive clones were found with a frequency of about 10^{-3} Cb^r Tc^s transformants, and one of these clones, designated 56C1, was selected after retesting (see Fig. 2) for further studies.

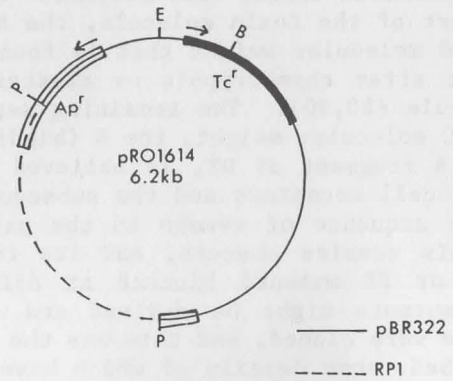


Fig. 1. Plasmid pRO1614, constructed from the insertion of a 1.85-kb segment of RP1 carrying replication genes, into the PstI site of pBR322 (24). This segment also carries the carboxy-terminal region of the β-lactamase gene of Tn1, and is oriented so that pRO1614 retains ampicillin resistance.

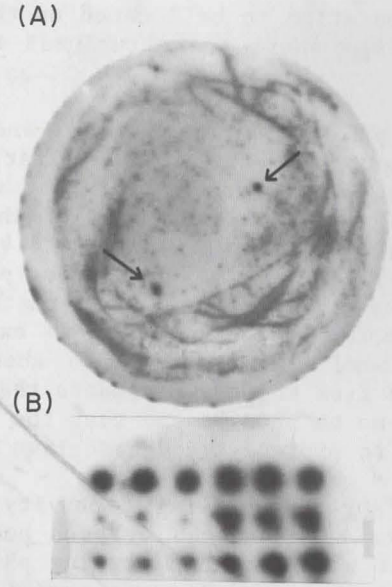


Fig. 2. Identification of antigen-positive clones by the filter-immune assay. (A) Initial identification of antigen-positive clones. Two such clones are indicated by arrows. (B) Verification of antigen-positive phenotype of clone 56C1. Top row (left to right): first 3 spots, Ps. aeruginosa PA0286: second 3, Ps. aeruginosa PA103. Middle row: first 3, E. coli HB101(pRO1614); second 3, HB101(pRC345). Bottom row: first 3, MAM2(pRO1614), second 3, MAM2(pRC345).

56C1 was found to carry a plasmid of about 23.9 kb, and, thus, an inferred 17.7 kb insert. Plasmid DNA from 56C1, designated pRC345, was then used to transform a number of nontoxigenic (Tox) mutants of Ps. aeruginosa PA0286. PA0286 is an auxotrophic mutant of another toxin-producing strain of Ps. aeruginosa, PA01 (32), the Pseudomonas strain on which most of the genetic studies of this species have been carried out. Nontoxigenic mutants of PA0286 were derived by nitrosoguanidine mutagenesis (1) and preliminary screening by the Elek immunodiffusion test (2). They were then tested for the lack of additional auxotrophic mutations, and finally for the CRM^- phenotype, that is, for colonies that, after lysis, failed to show the presence of products that would cross-react with antibodies to PE. All of 4 such CRM^- mutants were shown by the dot-blot test (Fig. 2) to be complemented to produce a CRM^+ phenotype when transformed with pRC345 plasmid DNA. The mutation in one of these mutants, designated MAM2, was then mapped after the MAM2 strain had been converted to a genetic donor by transfer of the Psedudomonas chromosomal-mobilizing factor, R68.45 (10,11). These mapping stud-

ies showed the MAM2 mutation to be located on the PAO chromosome in the 85 min region (29), where the structural toxA1 gene has been mapped (12) (Tab. 1).

The activity of pRC345 in E. coli HB101 and in MAM2 was investigated using 2 other procedures. The first procedure involved Western-blot analysis of proteins (5,34) that were immunologically reactive with anti-PE toxin on the basis of their size (17) (Fig. 3). Neither bacterial host carrying the pRO1614 vector produced detectable bands, but either host, when carrying pRC345, gave positive results. In both cases, a series of cross-reacting bands was found with a combined molecular weight grossly in excess of that of the toxin, the largest band corresponding to about 50,000 molecular weight. We concluded from these experiments that we had cloned only a part of the toxA gene on pRC345, and that the product of this gene fragment was subject to proteolytic degradation in both hosts.

The enzymatic (ADP-ribosylating) activity associated with PE (7) was also found to be expressed by both hosts carrying pRC345, and again the control host strains carrying pRO1614 were negative. The PE specificity of this activity was verified by its removal in the presence of antitoxin (Tab. 2). Moreover, the wheat-germ fraction containing EF2 to which the radioactivity was transferred was shown by polyacrylamide-gel analysis to have a molecular weight of

Tab. 1. Mapping the CRM$^-$ mutation in Ps. aeruginosa MAM2.

Recipient[1]	Selected Marker	% Coinheritance of tox allele: CRM$^-$ MAM2	% Coinheritance of tox allele: toxA1[2]
PAO949	pur-67$^+$	25	25
PAO949	cys-59$^+$	NT	10
GMA253	hisI 5075$^+$	5	NT
PAO944	cys-54$^+$	14	28

1) The chromosome-mobilizing plasmid R68.45 was transferred to MAM2 (met-28 trp-6 CRM$^-$) and this donor was mated with one of the recipients (31,32) as shown. 100 colonies prototrophic for the selected marker were selected and tested for transfer of the CRM$^-$ phenotype.

2) Linkage data of Hanne et al. (12).

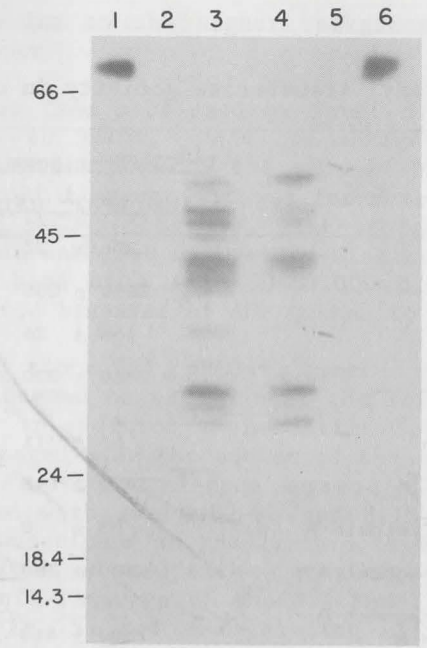


Fig. 3. Western-blot hybridization analysis of exotoxin A-related polypeptides expressed by clone 56C1. Lane 1, purified exotoxin A (0.2 µg); 2, E. coli HB101(pRO1614); 3, HB101 (pRC345); 4, P. aeruginosa MAM2(pRC345); 5, MAM2 (pRO1614); 6, exotoxin A (0.2 µg). Positions of protein molecular weight standards (BSA, 66,000; egg albumin, 45,000; trypsinogen, 24,000; β-lactoglobulin, 18,400; and lysozyme; 14,300) after electrophoresis are indicated (molecular weight x 10^{-3}).

about 90,000, in good agreement with the size of EF2 (16). These results appear to justify our conclusion that pRC345 carries a part of the structural toxA gene.

pRC345 DNA was found by restriction-enzyme cleavage to carry approximately 17.7 kb of PA103 chromosomal DNA, comprised of 8 BamHI fragments (Fig. 4). Several other antigen-positive clones like 56C1, each derived from a separate ligation and transformation procedure, were isolated during the preliminary screening and all were found to carry a similar spectrum of fragments. We, therefore, concluded that there appeared to be some barrier, either to the cloning, or to the expression of the intact toxin gene in E. coli using pRO1614. Similar cloning experiments were then undertaken using the Ps. aeruginosa MAM2 strain as a recipient. We were unable to identify any antigen-positive clones by our screening procedures, which we believe is due partly to the lower efficiency of transforming

Tab. 2. ADP-ribosyl transferase activity in cell extracts.[a]

Sample	[^{14}C]-ADP-ribose incorporated into EF-2[1] (CPM ± S.D.)
HB101(pRO1614)	256 ± 33
HB101(pRC345)	2640 ± 500
MAM2(pRO1614)	168 ± 26
MAM2(pRC345)	2060 ± 520
MAM2(pRC345) + anti-exotoxin A	61 ± 11
Exotoxin A	3470 ± 145
Exotoxin A + anti-Exotoxin A	55 ± 8
Diphtheria toxin fragment A	6500 ± 380
Diphtheria toxin fragment A + anti-exotoxin A	7500 ± 410

[a] All assays were done in triplicate. Samples treated with anti-exotoxin A were incubated with the IgG fraction (114 µg) for 10 min at 24°C prior to addition of urea (see Materials and Methods). The specificity of the inhibitory effect of anti-exotoxin A on the activity of exotoxin A was tested in controls by measuring the effect of the anti-exotoxin A on the ADP-ribosyl transferase activity of fragment A of diphtheria toxin, which is not antigenically similar to exotoxin A, but which has the same enzymatic activity as exotoxin A. Since anti-exotoxin A did not interfere with the activity of fragment A, the effect of the antitoxin on the activity of exotoxin A was specific.

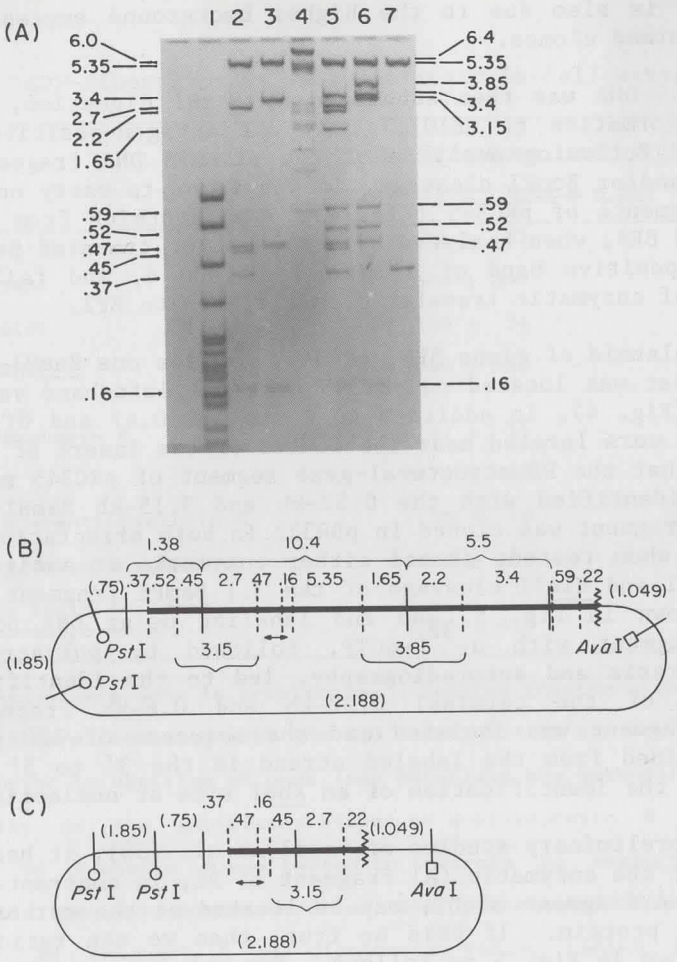
[1] The component in the Ef-=2 fraction of wheat germ to which radioactivity was transferred was shown, by polyacrylamide-gel electrophoresis, to have a molecular weight of about 90,000, in good agreement with published values for EF-2 (16) (data not shown).

MAM2, but is also due to the higher background expression of the nontransformed clones.

pRC345 DNA was then subcloned by BamHI digestion, religation, and transformation to HB101. Only one antigen-positive clone was isolated. Following analysis of the plasmid DNA fragments derived by BamHI and/or EcoRI cleavage, it was found to carry only 3 of the BamHI fragments of pRC345 (Fig. 4). The proteins from this clone, designated 8E4, when analyzed by Western-blot transfer produced only a single positive band of a size 30-35,000 d, and failed to show evidence of enzymatic transfer of ADP ribose to EF2.

The plasmid of clone 8E4, pRC346, carries one BamHI-fragment of 3.15 kb that was located on pRC345 near the left-hand vector insert junction (Fig. 4), in addition to 2 smaller 0.47 and 0.16 kb fragments that were located near the center of the insert of pRC345. We conclude that the PE structural-gene segment of pRC345 must, therefore, be identified with the 0.52-kb and 3.15-kb BamHI fragments. The 3.15 fragment was cloned in pBR322 in both orientations, neither of which, when tested, showed either enzymatic or antigenic activity. EcoRI and HinfI cleavage of the 3.1 BamHI fragment led to the map as shown in Fig. 5, and end labeling using DNA polymerase I Klenow fragment with α-^{32}P-dGTP, followed by polyacrylamide-gel electrophoresis and autoradiography, led to the identification and separation of the terminal 0.22-kb and 0.6-kb fragments. The 0.22-kb fragment was isolated and the sequence of 120 nucleotides was determined from the labeled strand in the 3' to 5' direction, leading to the identification of an XhoI site at nucleotide 39.

From preliminary studies of Vasil et al. (38), it has been concluded that the enzymatic (A) fragment of PE, in contrast to the corresponding A fragment of DT, may be located at the carboxy-terminal end of the protein. If this be true, then we can rationalize our data as shown in Fig. 5 as follows. For unknown reasons, there appears to be a barrier to the expression of the intact toxA gene on this plasmid vector, whether in E. coli or Ps. aeruginosa. In pRC345, we conclude that we have cloned the carboxy terminus of PE, which is located on the 520 and 450 nucleotide fragments. Expression appears to be due to the P2 tetracycline promoter of pBR322, located at approximately 45 nucleotides from the EcoRI site (4), and leading to the initiation of a hybrid protein from the ATG initiation site of the tetracycline-resistance gene, located 86 nucleotides from EcoRI (27). Such a hybrid protein would comprise 75 amino acids of the N-terminus of the tet protein, corresponding to a molecular weight of approximately 11,000 d, which would be fortuitously in frame with approximately 39,000 d of the carboxy terminus of the PE toxin, and would be consistent with the C-terminus of the gene being close to the EcoRI site at the right hand terminus of the 450 nucleotide fragment. The inability to isolate antigen-positive



clones carrying larger segments of the gene could be due to other upstream Sau3A/BamHI junctions being out of frame with the N-terminal fragment of the tet protein. We conclude that antigenic activity by pRC346 may also be due to expression promoted from P2, but with translation initiated within the 470 nucleotide fragment. The 30 to 35,000 d peptide thus produced would be translated from less than half of the toxA coding sequence than in pRC345, consistent with its retention of antigenic activity and loss of enzymatic activity.

The pRC345 plasmid is now being used as a probe to identify the amino terminus of the PE gene. Figure 6 shows a Southern blot of

Fig. 4. BamHI and EcoRI cleavage analysis of plasmid DNA from clones 56C1 and 8E4. (A) 5% polyacrylamide gel in Tris borate buffer of cleaved DNA fragments following electrophoresis at 25 V overnight. Lanes 1 and 4; HpaII cleaved pBR322 and EcoRI plus HindIII-cleaved λ DNA size standards, respectively. Lanes 2 and 3; plasmid DNA from clone 8E4 cleaved with EcoRI plus BamHI, or BamHI, respectively. Lanes 5 and 6; pRC345 DNA cleaved with EcoRI plus BamHI, or BamHI, respectively. Lane 7: pRO1614 DNA cleaved with EcoRI plus BamHI. The sizes of the smaller (<0.6 kb) fragments shown in kb are derived from the pBR322 standard on this gel. Sizes of fragments in kb from double digestion of pRC345 with BamHI and EcoRI (lane 5) are shown at left of gel, and those from pRC345 digestion with BamHI (lane 6) at right of gel. [The sizes of the larger (>1.6 kb) fragments are derived from the λ standard following agarose-gel electrophoresis.] (B) Order of the fragments on pRC345. The thin line represents the vector pRO1614, and the thick line represents PA103 chromosomal DNA. The symbols (|), (¦) and (⧘) represent EcoRI, BamHI, and Sau3A recognition sites, respectively (only one terminal Sau3A site on the insert and vector DNA being shown). The figures represent sizes in kb derived from cleavage analysis (6) as shown above, together with BamHI-cleavage data of the isolated EcoRI fragments (data not shown). The arrow between the 0.47 and 0.16 kb fragments indicates that their relative order is not established. The location of the AvaI and PstI sites and the sizes of fragments shown in parentheses are derived from published data of pBR322 (33) and pRO1614 (24). (C) Order of fragments on pRC346 derived from data shown in (A). For details see (B).

BamHI-cleaved PA103 chromosomal DNA, hybridized with ^{32}P nick-translated, BamHI-cleaved pRC345 DNA. It should be noted that the 520 bp fragment is absent in the PA103 digest, but an approximate 1.5-kb BamHI fragment shows homology. This finding would be consistent with the creation of the upstream cleavage site of the 520-bp BamHI fragment within the 1.5-kb BamHI fragment by ligation of a Sau3A terminus of PA103 DNA with the BamHI terminus of pRO1614.

We are currently using similar radioactive probes constructed from the 520 or 450-bp fragments to identify overlapping PA103 chromosomal fragments produced by other restriction enzymes, to ligate such fragments into pRO1614 and to screen for their clones by hybridization with the radioactive 520 or 450 bp fragments. In this way we hope to clone extensive upstream fragments of the toxA gene, irrespective of their ability to express antigenicity, in E. coli or Ps. aeruginosa, and then, if necessary, to explore expression of these fragments when coupled to expression vectors of various kinds.

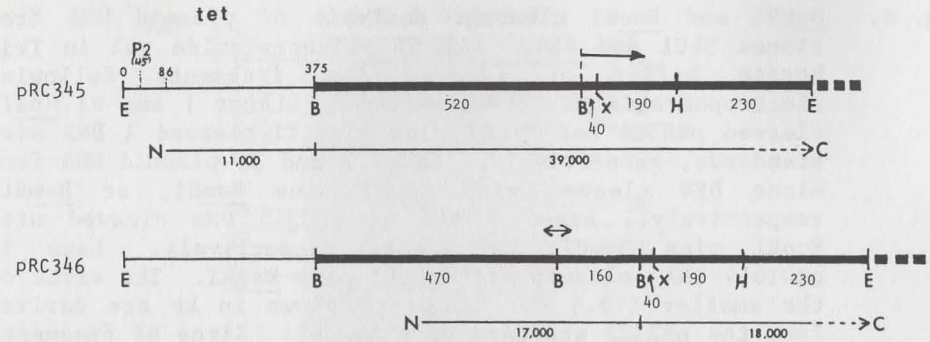


Fig. 5. Part of the toxA structural gene as cloned in pRC345 and pRC346. The thin line shows the EcoRI/BamHI segment of pRO1614 and the thick line shows PA103 chromosomal DNA. B, E, X, and H represent cleavage sites of BamHI, EcoRI, XhoI, and HinfI, respectively. The numbers above these lines represent base pairs measured rightwards from the EcoRI site of pRO1614. The numbers below these lines represent distances between cleavage sites in base pairs. P2 and tet represent the P2 (tet) promoter and part of the tetracycline-resistance gene of pRO1614. The single arrow indicates the region over which the base sequence has been determined. The double arrow indicates the alternative arrangements of the 470 and 160 nucleotide fragments. The lower line in each case terminated by N and C represents the region presumed to be translated, N representing the amino terminus of the hybrid protein and C the carboxy terminus including the toxA-encoded sequences. The numbers under these lines represent the calculated size of the part of the hybrid protein product in daltons.

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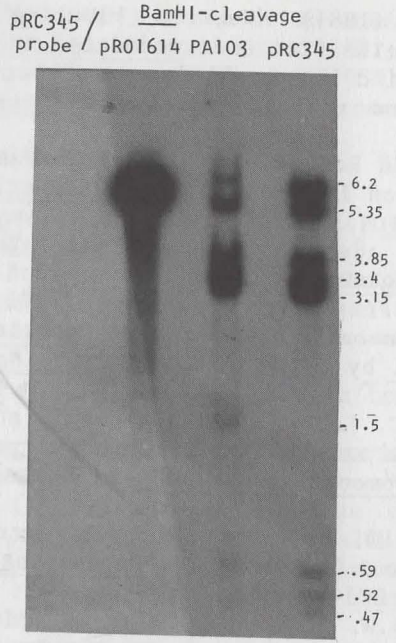

Fig. 6. Southern blot of hybridization with radioactive pRC345. BamHI-cleaved DNAs were separated on an 0.8% agarose gel for 13 hr at 20 volts. They were then transferred to a nitrocellulose sheet and hybridized with 1 μg, α-^{32}P nick-translated EcoRI-cleaved pRC345 DNA ($3x10^6$ c.p.m.). Slot 1, pRO1614 (10 ng); Slot 2, PA103 chromosomal DNA (20 μg); Slot 3, pRC345 (5 ng).

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STRUCTURE, FUNCTION, AND REGULATION OF THE PLASMID-ENCODED HEMOLYSIN DETERMINANT OF ESCHERICHIA COLI

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INTRODUCTION

In 1967, H. William S. Smith first reported on the extrachromosomal nature of hemolysin formation in Escherichia coli (1). Shortly thereafter it was demonstrated that transmissible plasmids carrying the information for the hemolytic phenotype were present in E. coli strains which were isolated from among the fecal flora of various animals. Since then a large number of hemolytic (Hly) plasmids have been characterized (2-5), clearly establishing the early assumption of the extrachromosomal nature of this property. These Hly plasmids belong to various incompatibility groups although the incJ2 and FVI groups are predominant. Their molecular sizes range from 60 kilobases (kb) to over 160 kb (2-5). As we have shown by hybridization with cloned hly genes, even those Hly plasmids which are structurally rather unrelated share extensive homology in their hemolysin determinants, suggesting that the hemolysin determinant may represent a discrete genetic entity that can undergo recombination without extended sequence homology. Association of the hemolytic phenotype with other known extrachromosomal phenotypes of E. coli such as antibiotic resistance, heavy metal resistance, enterotoxin production, or colonization factor antigens appears to be very rare and has never been observed in our laboratory. Recently it became apparent, however, that most hemolytic E. coli strains isolated from humans, especially from patients suffering from urinary tract infections, bear the hemolysin determinant on the chromosome (6,7). It was shown in several animal model systems (8,9,20) that chromosomal hly-determinants contribute to virulence whereas little effect could be demonstrated for plasmid-encoded hly-determinants.

In this chapter we will primarily describe the structural and functional aspects of a plasmid-encoded hemolysin determinant derived from the Hly plasmid pHly152 (3,10). In the last section we will compare our knowledge obtained on this determinant with recent data from chromosomal hly-determinants.

Structure of the Plasmid-Encoded Hly Determinant

The region of the low copy Hly plasmid pHly152 essential for complete hemolysis, e.g., synthesis of active hemolysin and its excretion, was cloned as a 14-kb SalI-partially HindIII digested DNA fragment into pACYC184. The resulting high-copy plasmid, pANN202-312, expressed in E. coli K12 similar amounts of external and internal hemolysin as the original pHly152. The same fragment was also cloned into the mobilizable broad host-range vector pKT231, and the resulting recombinant plasmid, pANN202-419, was introduced with RP4 into several gram-negative bacteria including Salmonella typhimurium, Aeromonas hydrophila, Proteus mirabilis, Pr. morganii, and Serratia marcescens. Hemolysin was expressed in all these hosts and properly excreted, indicating that the genetic information for the hemolytic phenotype was plasmid-inherited and located on the cloned HindIII-SalI fragment.

The region essential for hemolysin synthesis and excretion was further narrowed down by transposition of Tn5 or Tn3 into pANN202-312 and selecting for mutants unable to form hemolytic zones on blood agar plates (Hly^-). A region of about 7 kb, starting a few hundred base pairs (bp) to the left of the single Sma site (at coordinate 1.3 kb) and ending shortly before the PstI site (at coordinate 8.8 kb) as shown in Fig. 1, was found to be indispensable for the full hemolytic phenotype. The Hly^- mutants obtained could be divided into 2 groups (10), those which still produced internal hemolysin but were unable to secrete it (Tn3 insertions between coordinates 5.5 kb to about 8.6 kb) and those which were unable to synthesize hemolysin (Tn3 insertions from coordinate 1.0 to 5.5 kb). By complementing of both types of Tn3 mutants with cloned fragments of the hly region, we could identify 4 complementation groups. Two cistrons were shown to be essential for synthesis of active hemolysin (hlyC and hlyA) and 2 other cistrons were found to be required for excretion of hemolysin ($hlyB_a$ and $hlyB_b$) (10,15).

In addition to the coding region of the 4 cistrons (hlyC, A, B_a, and B_b) most Hly plasmids additionally seem to share homologous flanking regions. On 4 out of 5 Hly plasmids tested we found a region at the left end (proximal to hlyC) of more than 1 kb extending beyond the N-terminal end of the hlyC gene (12). Similarly all 5 Hly plasmids shared a common region at the right end of the hly-determinant (proximal to $hlyB_b$). Probes from both flanking regions were prepared and hybridized with the chromosomal DNA of E. coli K12 and hemolytic uropathogenic E. coli strains. Both probes hybridized

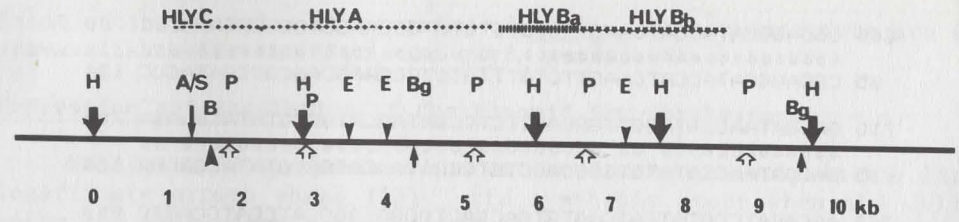

Fig. 1. Restriction map and localization of the 4 hly genes of the hemolysin determinant of plasmid pHly152. The map starts at the left (coordinate 0.06 kb) end with the HindIII site located in IS2 and ends with a HindIII site (coordinate 9.7 kb) outside of the other flanking sequence, ISx (see text for details). B (BamHI); Bg (BgIII; E (EcoRI); H (HindIII); P (PstI); A/S (AvaI/Sma).

with several chromosomal restriction fragments indicating that they may represent short, frequently occurring sequences (11,12). The hybridization patterns obtained with the 2 probes were different, however, suggesting that the 2 flanking sequences are not identical. This was further demonstrated by the complete lack of hybridization when 1 probe was tested against the other (12). DNA from the left flanking region was sequenced starting at the HindIII site at 0 kb (Fig. 1), a restriction site which is conserved in 4 out of 5 Hly plasmids. Comparison of a 216-bp sequence from this HindIII site to the right with the known sequences of the IS elements of E. coli revealed great similarity to sequence 876 (HindIII) to 660 of IS2 (Fig. 2). There are a few deviations with the reported IS2 sequence (21) which we cannot explain at the moment. The restriction map of this region fits that of IS2. These data clearly indicate that the hlyC proximal sequence is nearly identical to IS2. The other flanking sequence is localized between the PstI site at 8.8 kb and the BglII site at 9.6 kb (Fig. 1). Sequencing was performed from both restriction sites. The obtained sequences could not be correlated to any of the known IS elements. No clear-cut short repeats at the ends of this flanking sequence have yet been identified, suggesting that this sequence may be either partially deleted or may not represent a bona fide IS element.

The coding frame of the hlyC gene was revealed by fusion of the lacZ gene into the single BamHI site (13). The resulting fusion protein possesses a molecular weight of 146 kilodaltons (kd) and contains β-galactosidase activity, indicating that the N-terminal end of hlyC must be located about 360 bp upstream of the BamHI site. The DNA sequence of this region (Fig. 3) shows a single open reading frame starting with an ATG codon at the expected position. The size of this coding region (170 amino acids) matches the size of the HlyC protein (18,000 d) which was detected in mini- and maxi-cells of E. coli (14).

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660 CGCAGGCATACCATCAAGTTCTGCCTGTCTGCGAAGCAGCGCCCATACCC 709
    ööööööööööööööööööööööö  ööööööööööööööööööööööööö
 85 CGCAGGCATACCATCAAGTTCTGTTTGTCTGCGAAGCAGCGCCCATACCC 134

710 GACGATAACCATACGTTGGCAGCTCTCCGATAACATGGTGTATACGGAGA 759
    ööööööööööööö öö ööööööööööööööööööö ööööööööö öö
135 GACGATAACCATATGTGGGCAGCTCTCCGATAACATGATGTATACGGCGA 184

760 AGCACATCCGTATCATCAGTGTGACGACTGCGGCGGCCATCCATCCAGTC 809
    ööööööööööööööööö ööööööööö ööööööööööö ööö ööööööö
185 AGCACATCCGTATCATCCGTGTGACGGCTGCGGCGGCCGTCCTTCCAGTC 234

810 ATCGGTTCGTCTGAGAATGACGTGCAACTGCGCACGCGACACCCGGAGAC 859
    ööööööööööööööööööööööööööööööööööööööööööööööööööö
235 ATCGGTTCGTCTGAGAATGACGTGCAACTGCGCACGCGACACCCGGAGAC 284

860 AACGGCTGACTAAGCTT 876
    ööööööööööö ööööööö
285 AACGGCTGACGAAGCTT 301
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Fig. 2. DNA sequence starting from HindIII site (coordinate 0 kb in Fig. 1) 216 bp downstream compared to positions 876 to 660 of IS2 (21).

Upstream from the start codon for HlyC, a putative promoter sequence was identified with sequences fitting almost perfectly the -10 (Pribnow box) and -35 consensus sequences for E. coli promoters (Fig. 4). There is, however, only a rather short (15 bp) distance in between, which could explain the weak transcriptional activity of this promoter (see below). Further upstream of this putative promoter sequence is an AT-rich sequence which contains 2 12-bp direct repeats and several inverted repeats which overlap the directly repeated sequences. We are investigating the possibility that these sequences may be involved in the regulation of the hly determinant (see also below).

The region between the C-terminal end of hlyC and the N-terminal end of hlyA is 11 bp long. This region likely functions as a ribosome binding site since 7 bp are homologous to the 3' end of 16 S rRNA ("Shine-Dalgarno" sequence). The N-terminal end of hlyA was identified by microsequencing of a hlyA-lacZ fusion protein which was expressed under the control of λp_L and λp_R (Ref. 17; Vogel and Goebel, unpub. results). The coding region of hlyA was deduced from the size of the primary HlyA protein (106,000 d), of a truncated HlyA protein (104,000 daltons) (deleted at the PstI site at coordinate 5.1 kb) and of a HlyA-βgal fusion protein (220,000 d). The latter protein was obtained by fusion of lacZ into the BglII site at 4.3 kb. This fusion leads to a protein which has β-galactosidase activity and hence allows the determination of the reading frame of hlyA. The putative interspace between hlyA and $hlyB_a$ gene (Fig. 4) is 60 bp long and contains several stop codons in all 3 reading frames, and a new Shine-Dalgarno sequence beginning 10-bp in front of the putative start codon of $hlyB_a$. No sequence data are as yet available for the $hlyB_a$ and $hlyB_b$ genes.

Expression and Regulation of the Plasmid Hly Determinant

Hemolysin is synthesized and excreted most actively in the late logarithmic growth phase (13). Its synthesis stops when the cells enter the stationary phase. Aerobic conditions seem to be required for its expression and rich media stimulate both synthesis and excretion of hemolysin. These observations suggest a rather complex regulation of this protein. The native active hemolysin is highly unstable and this has hindered both its purification and efforts to investigate the regulation of its synthesis and transport. In bypassing this problem we have used insertions of Mud-1 phage (16) into pHly152 which allowed fusion of the lacZ gene to the hly-specific genes and subsequent assessment of gene expression along the hly-determinant. Furthermore we have constructed recombinant plasmids which carry genes where lacZ was fused either to hlyC or to hlyA.

It was previously shown (15) that Tn5 insertions in hlyC are polar, removing hlyA and $hlyB_a$ but not $hlyB_b$ activity, suggesting that the hly-determinant is transcribed from 2 promoters, one located in front of hlyC and the other near $hlyB_b$. The data obtained with Mud-1 insertions into pHly152 agree with this notion and allow determination of the strength of the transcription of the 2 promoters by measurement of the β-galactosidase activity. As shown in Fig. 5, the operon hlyC, A, B is transcribed rather weakly whereas the transcription of $hlyB_b$ is considerably stronger. The relative weakness of the hlyC proximal promoter was confirmed by cloning the HindIII-BamHI fragment (see Fig. 1) into pKO-11, a vector allowing identification of promoter strength by insertion upstream of the galK gene. The transcriptional activity observed (Tab. 1) was less than half that of the gal promoter, regarded as average in E. coli and slightly lower than $p\lambda_{C17}$, a phage λ promoter classed as "average to low." The hly promoter was, however, considerably stronger than $p\lambda_{482}$, a synthetic promoter determining the lowest transcriptional activity yet observed in this system.

The direction of transcription from the other hly promoter (in Fig. 4, transcribing $hlyB_b$) was determined by selection of Mu-sensitive derivatives from a Hly^- mutant containing the Mud-1 insertion in $hlyB_b$. Out of 6 Mu^s revertants, 5 were found to be Ap^r and exhibited the same level of β-galactosidase activity as the original clone but failed to produce internal hemolysin. This indicates that the deletion which affected the gene C-proximal end of Mu, and thereby caused the ts^+, Mu^s phenotype, extended into the adjacent $hlyB_a$ and hlyA genes. The direction of transcription from the $hlyB_b$ promoter is therefore from right to left, i.e., opposite to transcription of the hlyC, A, B_a operon. Restriction fragments con-

Fig. 3. DNA sequence of the hlyC region. The open reading frame fitting the size of HlyC protein starts at position 121 and ends at position 630.

taining this promoter have not yet been identified in the pKO-11 system. A polar effect from hlyC to hlyA is also observed when lacZ is fused to one of these genes. Whereas β-galactosidase activity of the HlyC-βgal fusion protein reaches 700 units, that of HlyA-βgal fusion protein is considerably less active (200 units); the copy numbers of the plasmids carrying the fusions are the same in both cases.

Hemolysin activity is not increased with increasing gene dosage, suggesting a negative control of transcription of the hly genes. However, when the dosage of the fused genes hlyC-lacZ or hlyA-lacZ is modulated by cloning these genes together with the control region (16) into the low copy plasmid pSC101, β-galactosidase activity drops in correlation to the gene dosage. This strict gene dosage effect of β-galactosidase occurs whether the other hly genes (hlyA, B_a and B_b for hlyC-lacZ and hlyB$_a$ and B_b for hlyC, hlyA-lacZ) were present or not, ruling out possible negative regulatory functions of these hly gene products. When lacZ is under the control of the hlyC proximal promoter, either as an intact gene (by Mud-1 insertion) or as a fusion gene with hlyC or hlyA, the level of β-galactosidase in contrast to hemolysin formation increases with the cell mass and reaches a maximum in the early stationary phase. This rules out the possibility that the observed decrease in internal and excreted hemolysin activity in the late exponential phase is caused by transcriptional (or translational) effects.

The cyclic nucleotides, cAMP and cGMP, had no effect on β-galactosidase activity in strains which carried lacZ placed under the control of the phlyC promoter indicating that hemolysin synthesis is not influenced by these compounds. There was, however, a remarkable difference in β-galactosidase activity in Mud-1 insertion mutants, with Mud-1 inserted either in hlyC or hlyA, when they were grown either aerobically or anaerobically. This effect correlates with the observed reduction in hemolysin formation under anaerobic conditions. No such effect was seen in expression of β-galactosidase activity when Mud-1 was inserted into hlyB$_b$. This suggests that promoter phlyC but not phlyB$_b$ is controlled on the transcriptional level directly or indirectly by the oxygen level in the cell.

Function of the Hly Gene Products

When pANN202-312 Δ H, a recombinant plasmid carrying exclusively the entire hly-determinant from HindIII (0.0 kb) to HindIII (9.6 kb) cloned into pACYC184, is transformed into minicells or maxicells of E. coli and the plasmid-specific gene products are

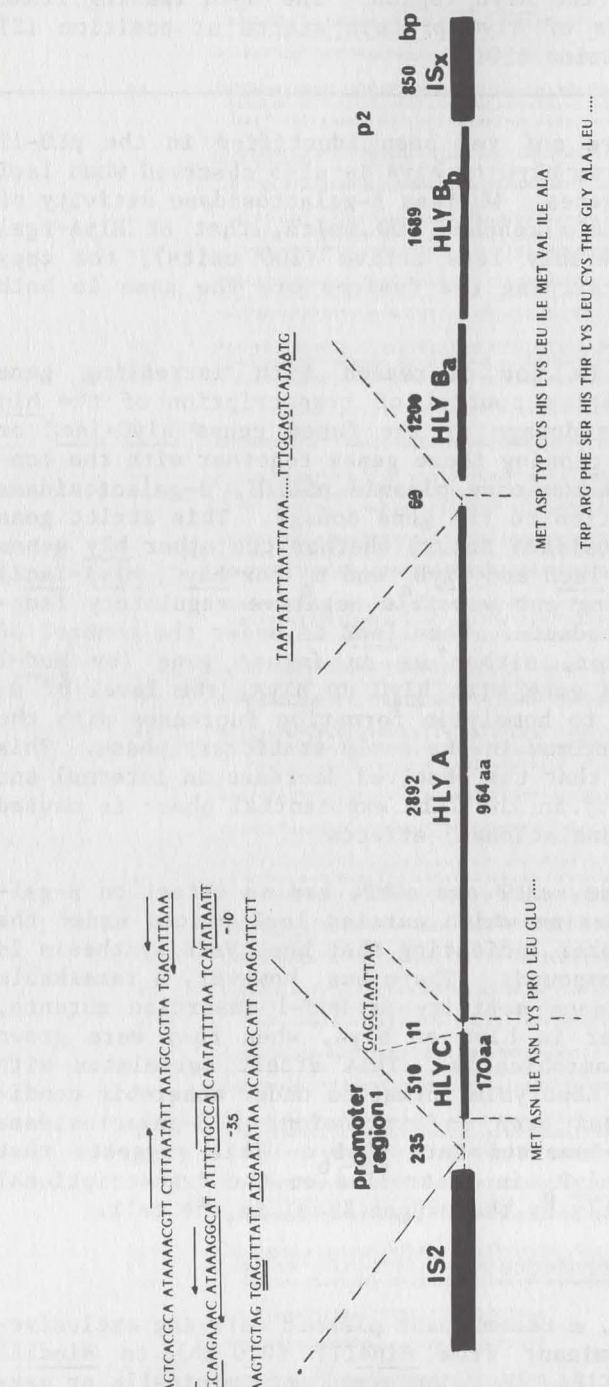

Fig. 4. Functional map of the hly determinant of pHly152 showing the interspace regions between hlyC and hlyA, and hlyA and $hlyB_a$, respectively. Characteristic sequences are specifically marked. N-terminal amino acid sequences deduced from the DNA sequences are shown for HlyC, HlyA, and $HlyB_a$.

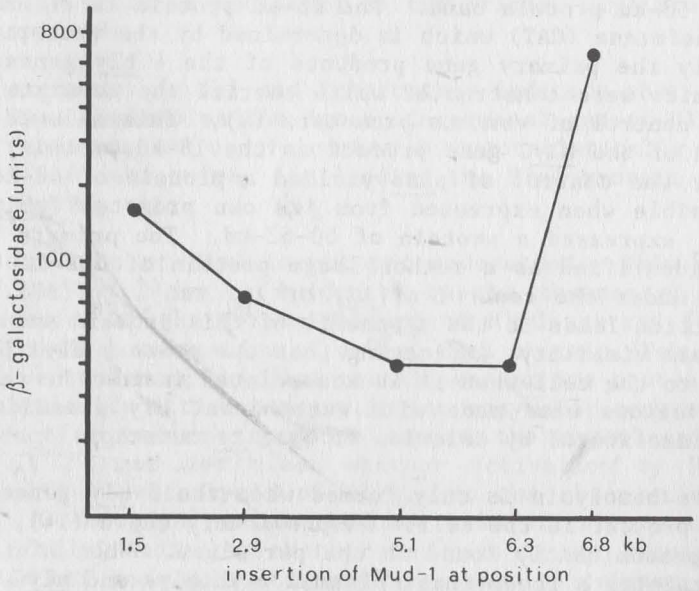

Fig. 5. β-galactosidase$_R$ activity after insertion of Mud-1 (Mu-lacZ, Y, Ap^R) into various rgions of the hly determinant. Positions of mud-1 insertions follow the coordinates in Fig. 1.

Tab. 1. Transcriptional activity observed by cloning the HindIII-BamHI fragment into pKO-11, a vector allowing identification of promoter strength by insertion upstream of the galK gene.

Promoter	Galactokinase activity*
pgal	1.000
$p\lambda_{C17}$	0.54
p hlyC	0.41
$p\lambda_{482}$	0.25
no insert	< 0.05

* Galactokinase activity (assessed as galactose phosphorylated per cell number and time) was neutralized to the E. coli promoter pgal takes as 1.0

labeled, 3 major proteins (18-kd, 25-kd, and 58-kd) are identified. In addition, there are several faint bands above (up to 106-kd) and below the 58-kd protein band. The 25-kd protein is chloramphenicol acetyltransferase (CAT) which is determined by the vector. In order to identify the primary gene products of the 4 hly genes, recombinant plasmids were constructed which carried the separate hly genes under the control of various promoters (4). This allowed the identification of the hlyC gene product as the 18-kd protein; the $hlyB_a$ gene under the control of plac yielded a protein of 48-kd which is barely visible when expressed from its own promoter (phlyC). Cistron $hlyB_b$ expressed a protein of 60-62-kd. The primary product of hlyA was identified as a rather large protein of 106-kd by putting this gene under the control of λ_{PL} or λ_{PR} and λ_{cts} (17). Temperature induction leads to the synthesis of this protein and to a rapid loss of cell viability, indicating that the primary HlyA product may be lethal to the cell when it is accumulated inside the cell. Similar observations were made with recombinant Hly plasmids in which hlyC was inactivated by deletion or by a ts mutation.

Active hemolysin is only formed when the 2 hly genes, hlyA and hlyC, are present in the cell. As previously shown (14), this hemolysin is predominantly found in the periplasm. When mini- or maxicells containing a recombinant plasmid with hlyA and hlyC are labeled with ^{35}S-methionine, the HlyC (18-kd) protein and a protein of 58-kd are the major hly-specific proteins (the vector-specific CAT protein bands at 25-kd). There are, in addition, small amounts of the 106-kd protein and several discrete protein bands with sizes larger and smaller than the 58-kd protein (14). This suggests that the 106-kd protein is eventually proteolytically degraded to yield several intermediates and a major product of 58-kd. This could be more directly demonstrated by pulse chase experiments. Short pulse labeling of minicells containing hlyC and hlyA with ^{35}S-methionine leads mainly to the appearance of the 106-kd protein which upon chase with cold methionine is converted to the major 58-kd protein (14). This proteolytic process does not depend on the HlyC protein since a mutant which is temperature-sensitive in HlyC still shows this proteolysis of HlyA under the restrictive conditions (14) without yielding active hemolysin under these conditions. It is difficult to decide at the moment whether this proteolytic degradation of the 106-kd HlyA is an artifact of the systems used (mini- and maxicells) or whether proteolysis of HlyA may be necessary for transport of hemolytically-active protein. This latter possibility is based on the following experimental evidence: (a) Pulse labeled 106-kd HlyA protein is found in the cytoplasm. (b) Deletion of 60-bp at the C-terminal end of hlyA leads to a truncated protein of 104-kd which yields hemolytically active protein in the presence of HlyC protein, but its transport is drastically inhibited (14). (c) The above described HlyA-βgal fusion protein, which is expressed by a fusion gene consisting of lacZ fused to the BglII site (4.2 kb) which is close to the C-terminal end of the hlyA gene, remains as a

large protein of 220-kd in the cytoplasm (Tab. 2). (d) The N-terminal end of HlyA (Fig. 4) does not appear to be a signal sequence, which would be necessary if the primary HlyA protein itself were transported.

The arguments in (b) and (c) further indicate that the proteolysis of HlyA depends on the intact C-terminal end of HlyA and suggest that this process occurs either autoproteolytically or that the C-terminal end is required for leading the HlyA protein to its cellular processing site.

The function of HlyC is not yet understood. This protein remains in the cytoplasm as evidenced by the absence of an obvious signal sequence at the N-terminal end of HlyC protein (Fig. 4) and the cytoplasmic localization of a HlyC-βgal protein (Tab. 2). It is required for rendering the HlyA protein and/or its proteolytic products hemolytically active, suggesting an enzymatic conversion of the HlyA product(s) before transport across the cytoplasmic membrane occurs. It is not yet clear whether activation by HlyC occurs before or after proteolysis of HlyA, but the observation that the truncated 104-kd HlyA protein becomes hemolytically active in the presence of HlyC indicates that proteolysis is not required for the HlyC action nor does activation by HlyC seem to trigger proteolysis of HlyA.

Hemolysin is only excreted through the outer membrane of the E. coli cell when hlyC and hlyA are complemented by $hlyB_a$ and $hlyB_b$. The 2 gene products are predominantly found in the outer membrane

Tab. 2. Protein formation and β-galactosidase activity in plasmid recombination.

Plasmid	Protein formed	β-galactosidase activity (units)			
		External	Periplasmic	Membrane bound	Cytoplasmic
none	none	22	25	30	28
pANN651 (*)	native β-galactosidase	20	30	82	3.300
pANN653 (*)	HlyC-βgal	30	25	122	560
pANN654 (*)	HlyA-βgal	18	22	76	189

(*) These recombinant plasmids were previously described (13). Plamid pANN651 carries Mud-1 inserted into hlyC, pANN653 contains a fused hlyC::lacZ gene with lacZ being fused at the BamHI site and pANN654 carries a fused hlyA::lacZ gene and an intact hlyC; fusion of lacZ occurred in pANN654 at the BglII site at coordinate 4.3 kb

fraction (15), but a substantial amount of both proteins remains in the M-band and even in the cytoplasmic membrane fraction (L-band). It was recently shown (15) that mutants defective in $hlyB_a$ tend to accumulate more hemolysin in the periplasm, whereas $hlyB_b$ mutants concentrate most hemolysin in the outer membrane without releasing it into the surroundings. Thus, $hlyB_b$ mutants form small hemolysis zones around growing colonies on blood agar plates. This has led to a model which assumes that $HlyB_a$ protein fixes hemolysin to the outer membrane and turns it inside out, whereas $HlyB_b$ protein releases hemolysin from the outer membrane.

The recent isolation of a chromosomal mutation (18) that specifically inhibits excretion of hemolysin without affecting transport of periplasmic proteins or excretion of colicin E1, E2, or E3 suggests that specific cellular protein(s) may be necessary in addition to the hly-specific gene products, $HlyB_a$ and $HlyB_b$, to properly insert the specific hemolysin transport machinery into the cellular envelope.

Comparison of Plasmid-encoded and Chromosomal hly Determinants

Chromosomal hly-determinants were cloned from uropathogenic E. coli strains of 4 O-serogroups (O4, O6, O18, and O75). Hybridization with plasmid-encoded hly genes indicated extensive homology in the 4 structural hly genes which were shown to be also functionally exchangeable (7,9). Differences were seen by more precise restriction mapping of the various determinants, particularly in hlyA which was verified by partial DNA sequences of hlyA from hly018 and hly075 (Wagner, Juarez, and Goebel, unpub. results). These differences seem to be somewhat concentrated at the C-terminal half of hlyA. Obvious differences between the plasmid and the chromosomal hly determinants exist in the flanking regions. None of the chromosomal hly determinants is flanked by IS2 at the hlyC proximal ends although several IS2 elements were identified in the chromosomes of these strains (12). The other end sequence of the hly determinant ($hlyB_b$ proximal) was found to be located close to both ends of hly determinants from several hemolytic O6 strains without flanking them directly. Evidence was obtained that this sequence is involved in the frequent loss of hly determinants in such strains (11).

The most dramatic difference was, however, observed in the in vivo action in various animal model systems (8,9,20, Hacker et al., unpub. results). Whereas the hly determinant of plasmid pHly152 proved to be nontoxic, highly toxic effects were exerted by the chromosomal hly determinants, especially by those of two O18 and O75 strains (9). This difference cannot be explained by different expression of hemolysin of these determinants, although there is circumstantial evidence (12,20) that the activity of the hlyC-proximal promoters may be different between different hly determinants. But even when the same promoter (phlyC, from hly determinant of plasmid

pHly152) was used, the chromosomal hly determinants still exerted a much higher toxicity in the mouse model (9). Recent experiments in which mixed hly determinants between a chromosomal (O18) and a plasmid (pHly152) determinant were constructed seem to indicate that the major cause for the difference in pathogenicity of the hly determinants is the composition of hlyA and its proper combination with hlyC (Wagner and Goebel, unpub. results).

CONCLUSION

While our knowledge on the structure of the plasmid-encoded hemolysin determinant and its relationship to the chromosomal hly determinants of uropathogenic E. coli strains has been considerably widened, the understanding of how the 4 gene products of the hly determinant interact to bring about synthesis and excretion of hemolysin is still rather incomplete and several questions remain unanswered: What is the function of HlyC protein which seems to be absolutely necessary for the production of active hemolysin? Is the secreted hemolysin identical in size to the primary product of hlyA gene or is the proteolytic processing of HlyA observed in mini cells essential for excretion? What is the precise function of the 2 gene products, $HlyB_a$ and $HlyB_b$, which are required for the excretion of hemolysin? The latter question is of general interest since little is known of how proteins are transported across the outer membrane of gram-negative bacteria.

Finally it remains to be shown whether plasmid hly determinants can undergo recombination with the chromosome and vice versa, and how this might occur.

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pIAA, A VIRULENCE PLASMID IN PSEUDOMONAS SAVASTANOI

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INTRODUCTION

Bacteria are responsible for many diseases of plants; however, only a few of the many species encountered are capable of establishing a pathogenic relationship with plants. Certain specialized functions therefore, must allow such bacteria to become pathogenic in plants. One component of pathogenicity of bacteria is expression of virulence which can be assessed on plants by the severity of the primary systems produced. Virulence, therefore, is used here as a quantitative term to describe the factor(s) responsible for production of primary symptoms in diseased plants. In the case to be discussed, the primary symptom of the disease is formation of tumorous outgrowths by the infected plant. Virulence, therefore is assessed by the size of the plant tumors incited by different strains of the pathogen. The aim of these studies is the development of concepts on virulence and its expression by the bacterium, Pseudomonas syringae p.v. savastanoi, (p. savastanoi), which causes tumors to form on oleander and olive plants. Expression of virulence is associated with genes borne on a plasmid in this bacterium.

Formation of tumors is the host response to production and secretion by the bacterium of high concentrations of the phytohormones, indoleacetic acid and cytokinins. These compounds are natural plant hormones required for normal development. Under normal circumstances only small amounts of the compounds are needed and regulatory mechanisms in the plant assure that their production and delivery to the sites of action occur in keeping with the developmental needs of the plant. In the diseased state, production and

secretion of phytohormones by the pathogen by-passes the normal regulatory systems of the host. The resulting phytohormone imbalances cause the host tissues to respond by undifferentiated proliferation.

The work to be discussed concerns the role of a plasmid pIAA1 in indoleacetic acid (IAA) production and virulence of P. savastanoi on oleander plants. The plasmid bears iaaM and iaaH, genetic determinants for tryptophan 2-monooxygenase and indoleacetamide hydrolase, respectively, the enzymes involved in the conversion of tryptophan to IAA by the pathway: L-tryptophan → indoleacetamide → IAA.

Genes for IAA Production Are Plasmid Borne

The genes, iaaM and iaaH are borne on a plasmid, pIAA, in oleander strains. Loss of pIAA1 by curing attenuates virulence and results in loss of IAA production (1,2). Virulence is fully restored together with IAA production if IAA$^-$ mutants are transformed with pIAA1.

A 2.7-kb EcoR1 restriction fragment (fragment M) bearing iaaM was cloned from pIAA1 into the vector plasmid RSF 1010 to construct a recombinant plasmid, pLUC1. Transformation of Iaa-mutants of P. savastanoi with pLUC1 restores high levels of tryptophan 2-monooxygenase activity and partial virulence (3). The locus coding indoleacetamide hydrolase is located promoter distal from iaaM; insertional inactivation of iaaM by IS-elements causes polar mutations and prevents expression of iaaH (4). Thus, iaaM and iaaH are part of an operon in oleander strains.

Distribution of iaaM and iaaH

By selection for resistance to α-methyl tryptophan, IAA$^-$ mutants are readily isolated. These mutants either lack pIAA, have deletions in pIAA with loss of iaaM and iaaH, or have an insertion element, IS-51, in the iaaM locus. (3,4,5).

In all oleander strains examined thus far, iaaM and iaaH occur on a plasmid. However, at least 3 different forms of pIAA have been found: the 52 kb pIAA1 in California oleander strains 2009 and 2015; the 72-kb pIAA2 in Italian oleander strains (2,5); the 70-kb pIAA3, in California strain 1050. In contrast, the IAA genes in olive strains appear to be borne on the chromosome. Southern blot experiments with pIAA1 restriction fragments as probes reveal that plasmids pIAA1, pIAA2, and pIAA3 share considerable amount of homology (C. Palm and T. Kosuge, unpub. results). However, homology between pIAA1 and chromosome fragments of olive strains appears to be limited to 5 to 6 kb spanning iaaM and iaaH.

Properties of Tryptophan Monooxygenase and Its Role in Regulation of IAA Production.

The foregoing evidence stressed the importance of tryptophan monooxygenase in virulence expression and IAA production. To determine its properties, the enzyme was purified to apparent homogeneity (S. Hutcheson, T. Kosuge, unpub. results). It is a FAD-linked enzyme with a monomer weight of 62 kd. It is strikingly product-inhibited by indoleacetamide (apparent Ki 7μM) and inhibition is competitive with respect to the substrate L-tryptophan; it is less strongly inhibited by IAA (apparent Ki, 220 μM). Tryptophan production is also regulated by tryptophan inhibition of anthranilate synthetase (M. Smidt, unpub. results); but the inhibition is less strong (apparent Ki for tryptophan, 30 μM). Tryptophan pool size therefore can be limiting in the production of IAA. When grown in culture media containing L-tryptophan, the wild types of P. savastanoi accumulate 10-fold or higher levels of IAA than in the absence of tryptophan. Certain mutants designated "super producers" have larger tryptophan pool sizes and accumulate 2 to 3 times more IAA than parental wild types. However, both "super producers" and parental types, when grown in media supplemented with L-tryptophan, produce increased but nevertheless similar amounts of IAA. Thus, the "super producer" phenotype is expressed only under conditions of tryptophan limitation (1). The tryptophan pool size therefore is an important factor in regulating IAA production by the bacterium and regulation of tryptophan monooxygenase by competitive feedback inhibition of indoleacetamide helps assure conversion of tryptophan to IAA does not occur at the expense of conversion to protein. In this way the primary requirements for tryptophan metabolism are met before conversion to indoleacetic acid occurs.

Specificity of Tryptophan Monooxygenase

Tryptophan monooxygenase is not specific for L-tryptophan, however. The enzyme catalyzes the oxidation of several tryptophan derivatives such as 5-hydroxy-, 5-fluoro-, and 5-methyltryptophan, in addition to azatryptophan (S. Hutcheson and T. Kosuge, unpub. results). Although DL-mixtures were used as substrates in these cases, it is presumed that the L-isomer is oxidized. Oxidation of these tryptophan derivatives by tryptophan monooxygenase represents an effective detoxification mechanism since the compounds otherwise would be toxic to P. savastanoi. The tryptophan derivatives are potent false feedback inhibitors of anthranilate synthetase, and strains lacking tryptophan monooxygenase are inhibited by the compounds.

Loss of pIAA1 and Effects on Physiology

Loss of pIAA1 and IAA production does not affect the ability of P. savastanoi IAA$^-$ mutants such as 2009-3 to grow in host tissue at least up to 16 days and does not affect the ability of the mutant to

grow in culture (1). Indeed, the mutant grows as well as its parent under both conditions. Ability to grow in the host and expression of virulence therefore are separate phenomena and genes on pIAA1 are not concerned with the ability of the bacterium to grow in its host. However, it is not certain how well IAA⁻ mutants survive in nature. The availability of such mutants allows tests for survivability to be carried out under field conditions.

The loss of IAA production and virulence without loss of viability illustrates a common phenomenon displayed by many plant pathogenic bacteria and fungi, i.e., spontaneous loss of virulence without loss of viability in culture.

The rationale for relating expression of virulence with secondary metabolism is illustrated by these studies. Loss of plasmids concerned with specialized functions likewise illustrates the concept of the "genetic plasticity" associated with plasmid borne determinants. The mobility associated with plasmid borne determinants has not been investigated in this bacterium; thus the possibility that pIAA1 is self transmissible deserves to be investigated.

It is interesting that IAA genes are on pIAA in oleander strains but on the chromosome of olive strains (5). Since oleander strains generally infect and produce tumors on both oleander and olive plants while olive strains (at least in California) generally show preference for olive plants, location of genes on the chromosome may be associated with reduced host preference. Indeed, most plant pathogenic bacteria show high host selectivity, a major exception being Agrobacterium tumefaciens (6). Genes determining host range in this bacterium appear to be borne on the large plasmid (Ti) that is associated with virulence and oncogenicity (7). An important experiment that remains to be done is the ability of Ti-less strains of A. tumefaciens to grow or survive in host tissue.

We had earlier mentioned that strains with tryptophan monooxygenase activity are not inhibited by 5-methyl tryptophan, and loss of pIAA1 confers sensitivity to the tryptophan analogue. Thus pIAA1 functionally resembles plasmids bearing genes for antibiotic resistance. Moreover, antibiotic resistance genes are commonly borne on transposons. It has not been determined if iaaM and iaaH occur on a transposon.

Other Functions of pIAA1

Wild type oleander strains convert IAA to IAA-lysine (8). The enzyme requires ATP and a divalent metal cation for activity. P. savastanoi mutants such as 2009-3 when lacking pIAA1 are unable to produce IAA-lysine when administered IAA; IAA⁻ mutants, in which iaaM is inactivated by IS-elements such as IS-51 or IS-52, nevertheless produce IAA-lysine when grown in the presence of IAA (L. Glass and T. Kosuge, unpub. results). Olive strains, however, typically

do not produce the conjugate and accumulate high concentration of IAA in culture (Glass and Kosuge, unpub. results). The possibility exists that conversion to IAA-lysine may help control the free pool of IAA in the cell and the amount of IAA excreted into host tissue. Other functions for IAA-lysine synthesis may become apparent as this system is studied in more detail.

Is There A Common Basis for Tumorigenicity?

It is clear that cytokinins and IAA are involved in tumorigenicity of plant pathogens. The earlier work by Skoog and Miller (9) had demonstrated the role of cytokinins and IAA in regulating morphogenesis of tobacco tissue. Both elevated levels and the relative proportions of the two compounds are important in modulating tobacco callus morphogenesis. The relationship between cytokinin and IAA and crowngall tumor morphology has been demonstrated (10, 11, 12, 13, 14). Tumors on tobacco plants inoculated with A. tumefaciens mutants constructed by Garfinkel and Nester (1) were analyzed for cytokinins and IAA (10). Mutants inactivated in the tms 1 or tms 2 locus by Tn-5 mutagenesis caused production of tobacco tumors with shoots; those with Tn 5-inactivated tm 2 locus caused formation of rooty tumors with low cytokinin and high IAA contents. Recently, Schröder et al., (15) provided evidence that the Tms 2 gene encodes an enzyme catalyzing the conversion of indoleacetamide to IAA. The enzyme activity was found in crowngall tumor tissue and in E. coli cells transformed with a recombinant plasmid containing the tms 2 locus. The indoleacetamide hydrolase activity was absent in untransformed tobacco callus cells and in E. coli. Although the in vitro conversion of L-tryptophan to indoleacetamide has not been demonstrated, wild-type crown gall tumor tissue shows a phenotype exhibited by wild-type P. savastanoi cells, being resistant to 5-methyltryptophan and sensitive to α-methyltryphotphan. Further, the amino acid sequences of the coding region of the P. savastanoi tryptophan monooxygenase gene shares 67% homology with the amino acid sequence of the tms 1 locus of T-DNA (T. Yamada and T. Kosuge, unpub. results). Further, Corbin, Yanofsky, and Nester (unpub. results) have found hybridization between iaaM and tms 1 occurs under low stringency conditions.

The picture is emerging that there is common basis for tumorigenicity and the similarity between iaaM and the tms 1 locus raises interesting questions concerning the origin of the genes concerned with auxin synthesis.

Genetics and Evolution of iaaM.

The aforementioned similarities between tryptophan monooxygenase and lysine monooxygenase raises interesting questions concerning the evolution of tryptophan monooxygenase. Lysine monooxygenase and δ-aminovaleramide hydrolase function to initiate the catabolism of lysine as a source of carbon (16). They catalyze reactions func-

tionally similar to those catalyzed by tryptophan monooxygenase and indoleacetamide hydrolase. It is tempting to suggest that the tryptophan monooxygenase-indoleacetamide hydrolase system originated from a catabolic system for tryptophan. The loss of genes coding enzymes beyond indoleacetamide hydrolase could have occurred; iaaM and iaaH were conserved under selective pressure for factors conducive to parasitism (17).

Plasmids encoding catabolic functions are discussed elsewhere in this volume. The similarities between pIAA1 and plasmids conferring catabolic functions suggest that studies on plasmids with specialized functions will permit broad concepts to be developed concerning the basic biology and metabolic functions of these interesting replicons.

Recently, Akiyoshi and Nester (unpub. results) have conclusively demonstrated that the tmr locus of T-DNA encodes the transferase which catalyzes the formation of isopentenyl adenine ribotide from 5'-AMP and dimethylallyl pyrophosphate. T-DNA fragments bearing the tmr of locus show homology with a restriction endonuclease fragment from plasmids of P. savastanoi. (Corbin, Yanofsky, and Nester, unpub. results). The results would suggest further similarities exist between P. savastanoi and A. tumefaciens T-DNA genes in phytohormone synthesis.

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THE MOLECULAR BASIS OF PLANT CELL TRANSFORMATION BY AGROBACTERIUM TUMEFACIENS

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Crown gall is an uncontrolled proliferation of tumor cells incited by Agrobacterium tumefaciens on most dicotyledonous plants. To accomplish this, the bacterium transfers and integrates a portion (the T-region) of its tumor inducing (Ti) plasmid into plant nuclear DNA (1,2,3,4). The transferred DNA (T-DNA) is expressed in the transformed cell and encodes enzymes involved in the synthesis of amino acid condensation products called opines (5,6,7,8,9,10). This paper will report data which suggest that T-DNA alos encodes genes which specify the synthesis . . . also encode the synthesis of the phytohormones, Indole acetic acid, Isopentenyl adenosine, Isopentenyl adenosine 5' monophosphate, and Isopentenyl adenine.

Garfinkel et al. (11,12) and Ooms et al. (13,14) isolated Tn5 insertion mutations in the T-DNA region of the Ti-plasmid. They described three tumor morphology loci tms, tmr, tml (Fig. 1): 1.) tms mutants (tumor morphology shoot) incite tumors with shoots emerging from the center of the unorganized tissue (Fig. 1b); 2.) tmr mutants (tumor morphology root) incite tumors with roots emerging from the tumorous growth (Fig. 1c); 3.) tml mutants (tumor morphology large) incite tumors on Kalachöe leaves which are larger, greener, and more amorphous than those incited by the wild-type strain (Fig. 1d). Ooms et al. (14,15) suggested that the morphologies exhibited are associated with the level auxin and cytokinin found in the tumors, as addition of auxin to tms-induced tumors suppressed shoot formation. Subsequently, Akiyoshi et al. (16) measured the levels of auxin and cytokinin in tumors induced by tumor morphology mutants. They found that tms-induced tumors contained elevated levels of cy-

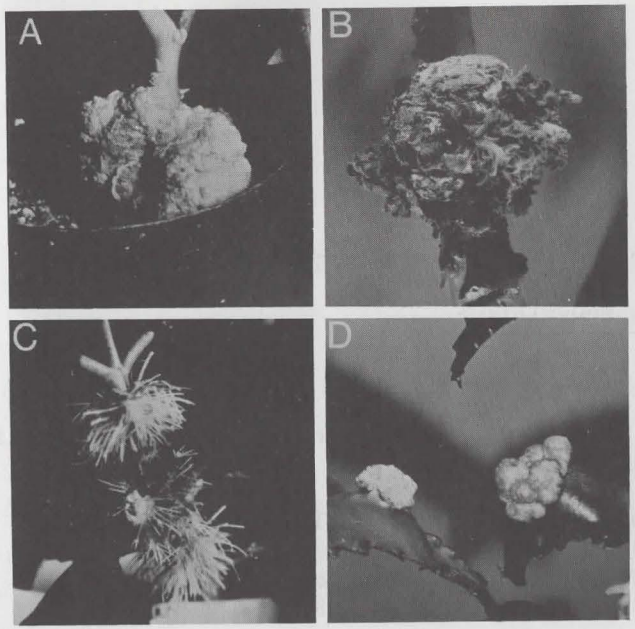

Fig. 1. Crown gall tumors: (A) A wild-type tumor incited by A. tumefaciens strain A6 on Datura stramonium stems. (B) A tumor on Nicotiana tabacum stem incited by a tms mutant of strain A6. (C) Tumors incited by a tmr mutant of strain A6 on the stem of Kalanchöe diagramontium. (D) Tumors incited by strain A6 (right) and a tml mutant of strain A6 (left) on leaves of Kalanchöe diagramontium.

tokinins and decreased levels of auxin while tmr-induced tumors had elevated levels of auxin and decreased levels of cytokinin when compared to wild-type tumors.

Willmitzer et al. (5) demonstrated that 2 polyadenylated transcripts are encoded by the tms loci. Schröder et al. (17,18) showed that the tms2 locus could be translated and transcribed in an Escherichia coli mini cell system to give a protein product of 49,000 daltons. This agrees with the protein product predicted by sequence data of the locus (19,20). The sequence data predict a protein product of 83,769 d for the tms1 locus. The sequence data of tms1 indicate a significant level of homology to the adenine binding region of p-hydroxybenzoate hydroxylase from Pseudomonas fluorescens suggesting that this protein binds adenine either as a substrate or as cofactor.

Schröder et al. (18) have demonstrated that the tms2 gene is expressed in both eukaryotic and prokaryotic systems. Extracts of

E. coli and Agrobacterium expressing the tms2 gene hydrolyzed indole-3-acetamide into indole-3-acetic acid while the same activity was not found in strains lacking this gene (18). They also demonstrated that extracts from crown gall cells exhibited an enzymatic activity which converted indole-3-acetamide to indole-3-acetic acid while no activity was detected in extracts of nontransformed cells. Inze and coworkers (21) showed that coinfection of tms1 or tms2 mutants with strains which contained only the tms2 or tms1 gene, respectively, produced wild-type tumors. These data show that diffusable gene products of the tms1 and tms2 genes can interact within the tumor cells to produce the same effect that external addition of α-naphthalene acetic acid produces, namely a wild-type tumor. The addition of α-napthalene acetamide to tumors incited by tms1 mutants restores the wild-type phenotype, suggesting that gene 1 encodes an enzyme involved in the synthesis of an intermediate in the production of the auxin indole-3 acetic acid. Thus, it can be concluded that the tms1 and tms2 loci encode enzymes involved in the synthesis of the plant phytohormone indole-3-acetic acid.

The correlation of shoot production with an elevated cytokinin to auxin ratio and the involvement of the tms loci with auxin production suggests that the tmr locus is involved with the production of cytokinin. This, indeed, is the case. Recently Akiyoshi et al. (22,23) have demonstrated that the tmr gene encodes an enzyme of cytokinin biosynthesis, dimethylallylpyrophosphate: AMP dimethylallyl transferase (DMAPP transferase). Initially they demonstrated DMAPP transferase activity in tobacco crown gall tumors incited by the strain A6 while no activity was detected in normal tobacco tissue (Tab. 1). This table also shows that DMAPP transferase activity is present in elevated levels in a tumor incited by a tms mutant of A6 (tms163) and is absent in a tumor incited by the A6 tmr mutant tmr149.

Recently, work by D. Akiyoshi in our laboratory has demonstrated DMAPP transferase activity in cell-free extracts of E. coli which contain a cloned tmr gene (23). The enzymatic activity is detected both when the gene is controlled by its own promoter and when it is fused to the lac promoter of pUC18 (24). Table 2 details the activity detected with the following T-DNA clones. pTiA6 EcoR1 fragment 7 was cloned into PUC 18 in both orientations. This fragment contains the tms1, tmr, and tml loci. To determine if the activity detected was due to the tmr locus or one of the other loci, clones were constructed which removed the other loci. EcoR1 fragment 7 cloned into pUC18 in orientation I, the direction of transcription of lac, tms1, tmr, and transcript 6a all being the same, was cut with the restriction endonuclease KpnI and then recircularized by ligation removing transcripts 6a and 6b of the tml locus to give clone pDQ14. The clone pDQ64 was constructed by taking EcoRI fragment 7 cloned into pUC18 in orientation II, cutting with the restriction endonuclease SalI and then recircularizing the molecule

Tab. 1. DMAPP transferase activity in tobacco crown gall tumors.

Plasmid	Enzyme Activity	
	Radioactivity in iP/iPA region (cpm)	Units*
Nicotiana tabacum var Xanthi Normal Callus	N.D.++	-
A6S/2+ tumor	8,950	7.6
tmr-149 tumor	N.D.	-
tms-393 tumor	31,830	27.2

* Units are defined as the synthesis of 1 fmol (^{3}H) isopentenyladenie/isopentenyladenosine per min. at 22°C (1 fmol = 13 cpm).

\+ N. tabacum cv White Burley. Other tumor lines were incited on N. tabacum cv Xanthi nc.

++ N.D. = not detected.

thereby removing the 5' end of the tms1 gene (25,26). The final construct, pRA120, was a fusion of the tmr gene to the α peptide of β-galactosidase. It was constructed by inserting a restriction fragment with a blunt end at the first nucleotide of the second condon of the tmr gene extending to the BamHI site between fragments 29 and 19 of pTiA6, into SmaI/BamHI cut pUC18. This plasmid was then cut with BamHI and SalI and ligated to a BamHI/SalI restriction fragment from a mutant A6 Ti-plasmid with a Tn5 insertion in transcript 6a within the BamHI fragment 19. The resulting plasmid contained the entire tmr gene linked to the first ten codons of the α peptide of β-galactosidase. The resulting protein has 10 additional amino acids at the amino terminus of the gene and should be under the regulation of the lac promoter on pUC18.

Enzyme activity was then measured in strains containing EcoRI fragment 7 cloned in the wide host range vector pRK290 (27) (Tab. 3). No activity was detected in extracts of a Tn5 insertion mutant within the tmr gene coding region (tmr149, Tab. 3). However, a low level of activity was detected in another mutant which gave a tmr

Tab. 2. Enzymic activity with T-DNA clones.

Plasmid	Enzyme Activity	
	Radioactivity in iP/iPA region (cpm)	Units*
pUC18	N.D.+	-
pUC18 EcoRI 7 (orientation I)	7,730	6.6
pDQ14	14,900	12.7
pUC18 EcoRI 7 (orientation II)	4,190	3.6
pDQ64	2,750	2.4
pRA120	10,100	8.6

* Units are defined as the synthesis of 1 fmol (^{3}H) iP/iPA per min at 22°C. (1 fmol = 13 cpm).

\+ N.D. = not detected.

phenotype on plants. When this mutant 169 was mapped, it was located 20 nucleotides past the termination codon for the protein but before the polyadenylation sites of the gene. Thus, these studies establish that the tmr gene produced is an enzyme involved in the biosynthesis of phytohormones with cytokinin activity.

From these studies, it has been established that the tms1 and tms2 loci encode enyzmes involved in the production of the plant phytohormone Indole-3-acetic acid and that the tmr locus encodes an enzyme of cytokinin biosynthesis. Thus, the transformation to a tumorous phenotype is the result of phytohormone production induced by the T-DNA which A. tumefaciens has inserted into the plant nuclear genome. The T-DNA also encodes the synthesis of compounds called opines, which can be utilized by the bacterium as sources of carbon and nitrogen creating a unique ecological niche for the bacterium. Therefore, the transformation of plant cells by Agrobacterium tumefaciens is a natural case of genetic engineering.

Tab. 3. Enzyme activity measured in strains containing fragments cloned in the wide host range vector.

Plasmid	Enzyme Activity	
	Radioactivity in iP/iPA region (cpm)	Units*
pRK290	20	<<0.1
pRK290-EcoRI7::Tn5(349)	10,820	9.3
pRK290-tmr-149:Tn5	80	<0.1
pRK290-tmr-169:Tn5	2,400	2.0

* Units are defined as the synthesis of 1 fmol (^{3}H) iP/iPA per min at 22°C. fmol - 13 cpm).

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POST-TRANSCRIPTIONAL REGULATION OF CHLORAMPHENICOL ACETYL TRANSFERASE

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QUESTIONS

This report describes some of our recent work which extends previous studies of post-transcriptional control of antibiotic resistance (8,9,10). Our previous efforts were directed towards elucidating the post-transcriptional mechanism by which erythromycin induces the synthesis of an N-methyl transferase that in turn, catalyzes the post-transcriptional methylation of adenine in 23S rRNA, a process that leads to reduced affinity between erythromycin and its target, the ribosome.

The central questions that we ask in these studies are: 1) How does the cell perceive the presence of the inducing antibiotic? i.e., What molecular species serves as the sensor of the antibiotic?, and 2) Upon sensing the presence of the inducer, how does the cell link the information to the process that ultimately increases gene expression? Our present studies examine these questions in relation to the chloramphenicol-induced synthesis of chloramphenicol acetyl transferase, an enzyme that confers resistance by inactivating chloramphenicol.

SUMMARY

The +1 site for initiation of inducible chloramphenicol acetyl transferase (CAT) messenger ribonucleic acid (mRNA) encoded by plasmid pC194 was determined experimentally using γ-^{32}P-ATP-labeled run-off transcripts partially digested with T1 ribonuclease. By partial digestion of the in vitro transcripts with S1-, T1-, and cobra venom nucleases as probes of mRNA conformation, single- and

double-stranded regions, respectively, were also identified. Thus, a prominent inverted complementary repeat sequence was demonstrated spanning the +14 to +50 positions which contain the complementary sequences CCUCC, and GGAGG (the Shine and Dalgarno sequence for synthesis of CAT) symmetrically apposed and paired as part of a perfect 12 bp inverted complementary repeat sequence (-19.5 kcal/mol).

The CAT mRNA was stable to digestion by T1 ribonuclease at the 4 guanosine residues in the Shine and Dalgarno sequence GGAGG, even at 60°C, suggesting that nascent CAT mRNA allows ribosomes to initiate protein synthesis inefficiently and that induction involves post-transcriptional unmasking of the Shine and Dalgarno sequence. Consistent with this model of regulation, we found that cells carrying pC194, induced with chloramphenicol (CAM), contain about the same concentration of pulse labeled CAT-specific RNA as do uninduced cells.

Induction of CAT synthesis by the nonacetylatable CAM analog fluorothiamphenicol was tested using minicells of Bacillus subtilis carrying pC194 as well as minicells containing the cloned pC194 derivatives in which parts of the CAT structural gene were deleted in vitro using Bal31 exonuclease. Optimal induction of both full-length (active) and deleted (inactive) CAT required similar concentrations of fluorothiamphenicol, whereas induction by CAM required a higher concentration for the wild-type full-length (active) CAT than for the (inactive) deleted CAT. Because synthesis of deleted CAT was inducible, we infer that CAT plays no direct role in regulating its own synthesis.

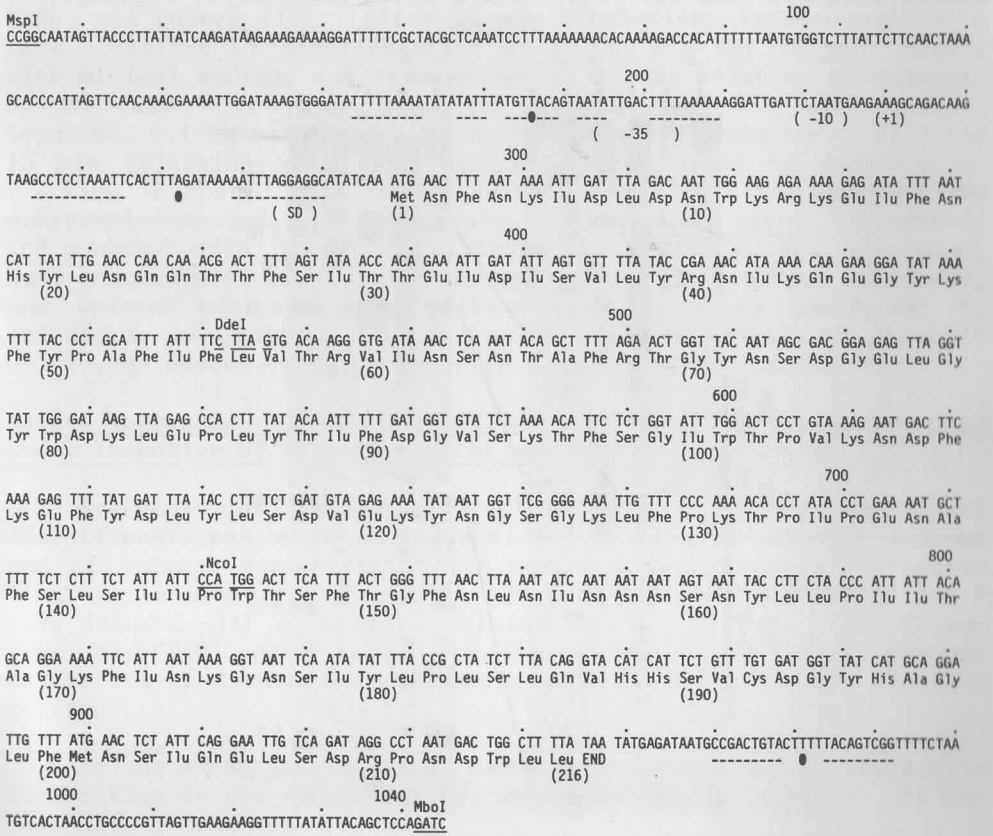
EXPERIMENTAL

DNA Sequence of the Structural Gene for CAT Including the Promoter and Putative Transcription Terminator

The -35 and -10 sequences have been identified on the basis of similarity to the respective consensus sequences in E. coli (17,20) and the +1 site for transcription initiation was determined experimentally in the present study. The structural gene sequence is flanked by an inverted complementary repeat sequence at the 5' end overlapping the CAT Shine and Dalgarno sequence and postulated to act as a post-transcriptional regulatory element, and by an inverted complementary repeat sequence at the 3' end postulated to act as a rho factor-independent transcription terminator. An additional inverted complementary repeat region overlapping the -35 region of the CAT promoter and of unknown function is also shown. Sites within the structural gene for digestion by DdeI and NcoI restriction endonucleases are indicated. Nucleotides are numbered above the text line and amino acids are numbered using unsigned bracketed numbers below the text line. Dashed lines indicate inverted complementary repeat regions. SD, denotes Shine and Dalgarno sequence.

Autoradiographic Analysis of Digestion Products Obtained Using In Vitro CAT Transcripts Partially Digested with Nucleases as Probes of Conformation

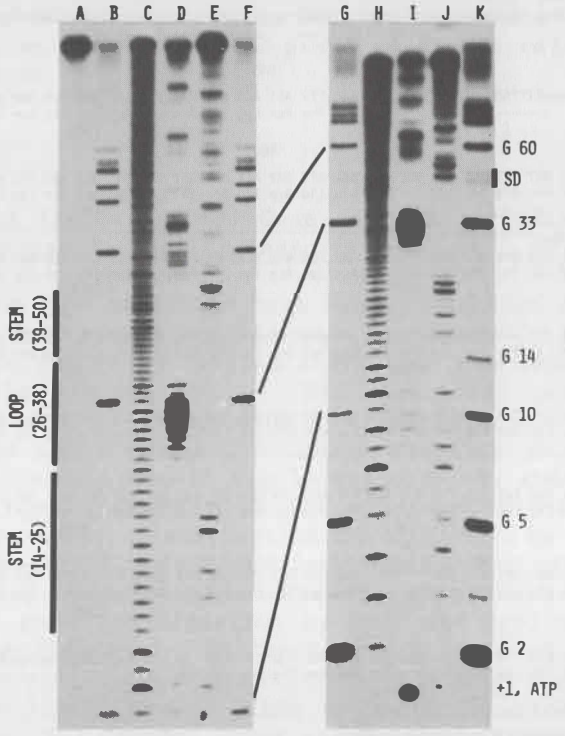
DNA template for transcription was prepared by digesting plasmid pC194 DNA sequentially with MspI and DdeI, yielding 5 fragments (1651, 609, 452, 160, and 40 bp in length) which were separated by electrophoresis on a 5% polyacrylamide gel. The third largest fragment, 452 bp in length, was located after ethidium bromide staining of the gel, electroeluted, and used as DNA template for in vitro synthesis of CAT mRNA transcripts. Synthesis of CAT mRNA transcript was performed in a 100 µl reaction mixture containing: 20 mM Tris HCl, pH 7.9; 5 mM $MgCl_2$; 100 mM KCl; 0.1 mM DTT; 0.1 mM Na_2EDTA; GTP, CTP, UTP, 0.3 mM each; 0.12 mM unlabeled ATP; γ-^{32}P- labeled ATP, 2.5 mCi, specific activity greater than 7,500 Ci/mM; and 6 µg E. coli RNA polymerase. Incubation was for 60 min. at 37°C. At the end of the incubation period 4 µl of 10% Na dodecylsulfonate (SDS) and an equal volume of stop buffer (9 M Urea; 25 mM Na_2EDTA; 0.1% bromophenol blue; and 0.1% xylene cyanol) was added. The sample was

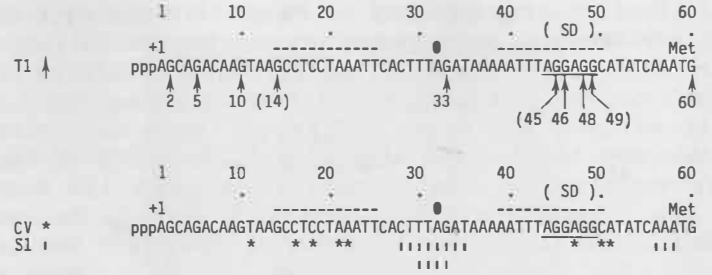


heated at 75°C for 5 min and was applied to a preparative polyacrylamide gel (8% acrylamide, 0.4% bisacrylamide) containing tris-EDTA-borate (TEB), (0.089 M tris base, 0.089 M boric acid, and 2 mM Na_2EDTA, pH 8.3).

The procedures described by Vournakis et al. 1981 (23) for analysis of end-labeled RNA transcripts were used. Lanes A-F were run on 10% polyacrylamide gel slabs to show structural features close to the CAT structural gene, whereas lanes G-K were run on 20% polyacrylamide gel slabs to show smaller fragments corresponding to structural features beginning at +1. (A) undigested control, (B,F,G, and K) T1 digests, (C and H) alkali digest standards, (D and I) S1 digests, (E and J) Cobra venom digests. Lane K contains the same sample shown in lane G, but overexposed to demonstrate the presence of 4 weak bands corresponding to the 4 inaccessible guanosine residues in the Shine and Dalgarno sequence.

The figure below summarizes, autoradiographic data presented above showing the location of transcription initiation based on T1 mapping of in vitro transcripts, and location of regions with conformation in accessible single-stranded- (T1- and S1-sensitive), or double-stranded form (CV- i.e., cobra-venom-sensitive), regions.





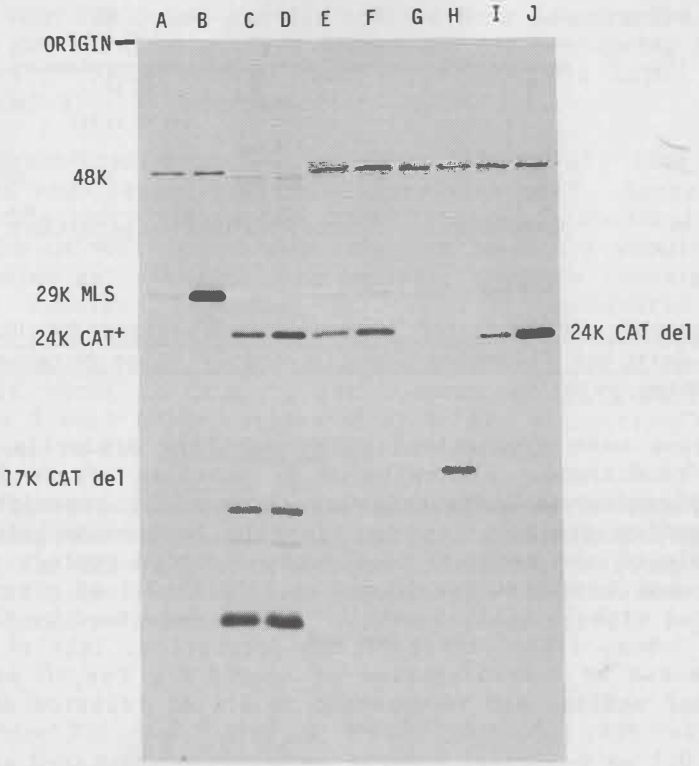
Autoradiographic Analysis of Proteins Synthesized by B. subtilis Minicells Carrying Plasmid pC194 DNA and Deletion Mutations in the CAT Structural Gene

Proteins were synthesized by B. subtilis minicells under the following conditions. Minicells of B. subtilis strain BGSC 1A197 carrying plasmids as indicated were prepared by centrifugation in sucrose density gradients as described by Mertens and Reeve (13). For analysis of the products of plasmid-directed protein synthesis, minicells were incubated for 30 min at 37°C in 0.5 ml minimal medium supplemented with 50 µCi ^{35}S-methionine, as described by Shivakumar, Hahn, and Dubnau (19). After 30 min incubation, labeled minicells were collected by centrifugation at 10,000 X g for 10 min, washed with minimal medium, and resuspended in 0.1 ml solution containing: 50 mM tris HCl, pH 8.0; 1 mM Na_2EDTA; and 25% w/v sucrose. Lysozyme, 0.1 mg was added and the mixture was incubated at 37°C for 15 min following which the minicells were lysed by addition of cracking buffer, and the resultant proteins fractionated by polyacrylamide gel electrophoresis. (A,B) pE194 cop-6, uninduced, and induced with 0.1 µg/ml erythromycin; (C,D) pC194, uninduced, and, induced with 0.3 µg/ml chloramphenicol; (E,F) pBW18, uninduced, and induced with 0.3 µg/ml chloramphenicol; (G,H) pBW18 del 9, uninduced, and induced with 0.3 µg/ml chloramphenicol; (I,J) pBW18 del 10, uninduced, and induced with 0.3 µg/ml chloramphenicol.

Autoradiographic Analysis of CAT and its Mutant Forms Synthesized Under Induction by Chloramphenicol and Fluorothiamphenicol

Synthesis of intact and deleted forms of chloramphenicol acetyltransferase by B. subtilis minicells as a function of inducer and of inducer concentration was next studied. Minicell preparations containing (A) pBW18; (B) pBW18 del 10; and (C) pBW18 del 9 were induced with a range of chloramphenicol (CAM) or fluorothiamphenicol (FTAM) concentrations at the concentrations shown. General conditions of incubation and sample workup were as in the previous section.

To the extent that protein bands other than CAT have comparable intensities in the autoradiogram, synthesis of the deleted CAT ap-



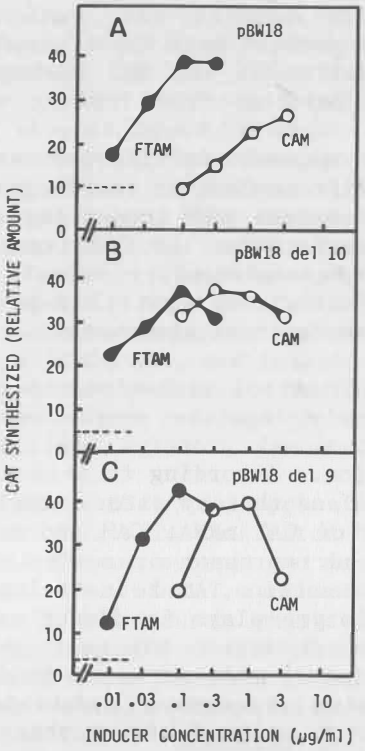
peared to be hyperinducible, first with respect to the lower background seen in the uninduced sample, and second with respect to the higher absolute intensity of the CAT band seen in the autoradiograms. A possible interpretation of this observation would be that since low (subinhibitory) concentrations of CAM were used for induction, the basal level of CAT expression in pBW18 inactivated part of the already low CAM concentration, whereas deleted forms of CAT were unable to do so for lack of enzymatic activity.

To test this hypothesis, we measured the ability of FTAM to induce CAT synthesis. FTAM can serve as a useful probe of induction because it contains $-CH_2F$ instead of $-CH_2OH$ at the 3-position and is therefore resistant to inactivation by the acetylation reaction which initially derivatizes the 3-OH position (21). In these determinations a series of inducer concentrations bracketing the minimum inhibitory concentration was used. As shown in the figure, induction by CAM, minimum inhibitory concentration (m.i.c.) = 10 µg/ml, progressively reached a maximum induction level in the 1-3 µg/ml range, whereas cells induced with FTAM (m.i.c.) = 1 µg/ml were maximally induced to a comparable level over the 0.01-0.3 µg/ml range.

If the apparent difference in inducing potency could be ascribed to acetylation of CAM, we would expect a shift in the concentration of CAM required for induction of (enzymatically inactive) deleted forms of CAT and no shift in the concentration of CAM required for induction. As shown in Fig. 5B and C for both the 24K deleted CAT (encoded by pBW18 del 10) and 17K deleted CAT (encoded by pBW18 del 9), this was found to be the case. We therefore infer that acetylation of CAM inactivates both antibiotic and inducing activities, and that both inducing activity and antibiotic activity are inseparable.

DISCUSSION

The 5' end of the CAT mRNA contains a 37-base inverted complementary repeat sequence, which in the associated form is capable of sequestering the translation initiation sequence of CAT. In this conformation the leader region would associate to form 12 perfectly matched sequential base pairs and experimental data obtained in this study indicate that the region possesses a high degree of thermal stability insofar as the 4 guanosine residues comprising the Shine and Dalgarno sequence are relatively unavailable for interaction



with T1 ribonuclease, even at 60°C. These findings accord with the free energy of association calculated by empirical formalisms (3,22) which yield an estimate of -19.5 kcal/mol, suggesting a high degree of thermal stability for this region of CAT mRNA.

Inverted complementary repeat sequences near the site of transcription initiation could serve as regulatory protein binding sites which, acting at the DNA level might function as a regulator of transcription. In fact, a prominent inverted complementary repeat region which spans residues 144 to 209, centering on residue 186 is shown in Fig. 1. This region overlaps the putative -35 sequence of the CAT promoter but does not extend into the messenger portion. Such a sequence could, in principle, serve as an operator for binding of a regulatory protein. However, at present we are unable to assign a primary regulatory function to this sequence. We feel that transcriptional control at the level of induction by CAM as the principal regulatory mechanism is unlikely because nascent CAT message synthesized after transcriptional induction would have a high probability of folding and assuming the stable inactive conformation shown in Fig. 8. Such a model would require 2 induction steps for regulation of CAT expression, one for transcriptional activation and one for translational activation. The simplest interpretation would be that regulation of CAT by CAM takes place at the level of translation.

A mechanism for induction must therefore describe how a comparatively stable association in the CAT message leader sequence is either disrupted or prevented from forming under inducing conditions. In the case of inducible erythromycin resistance (7,8,9), a translation initiation sequence is likewise masked and mRNA realignment is enabled by an intramolecular rearrangement of the erythromycin resistance rRNA methylase mRNA leader sequence. Unlike the control region for MLS resistance, the CAT regulatory region contains only a single inverted complementary repeat sequence with no apparent preemptor or associated control peptide characteristic of transcriptional or translational attenuators.

A second class of control mechanisms to consider, e.g.. mechanisms which autogenously regulate synthesis of phage T4 gene 32 product (5) or of ribosomal proteins (15), acts at the level of translational repression. According to this type of mechanism, CAT would bind directly to and thereby alter stability of the associated inactive conformation of CAT mRNA; CAM and CAT protein would thus function as inducer and repressor or activator, respectively. The various subcloning, resection, and insertion experiments suggest that the active CAT enzyme plays no direct role as a regulator of its own expression, which raises the question whether CAT protein plays any direct regulatory role at all. Studies of Lovett and coworkers (4,24,25) using a cognate inducible CAT from Bacillus pumilus subcloned into B. subtilis favor this idea. In their studies it was reported that trpC and DHFR inserted into the Bacillus pumilus CAT structural gene downstream from the CAT ribosomal binding site were inducible by CAM; on the other hand, DHFR inserted upstream from the ribosome binding site was expressed constitutively.

A further parallel in the comparison between induction of CAT and of MLS resistance is in the association between the activity of the antibiotic as inducer of resistance and activity as an inhibitor of protein synthesis. 3-deoxy CAM has been reported by Winshell and Shaw (26) to possess activity as a gratuitous inducer of CAT. Although the m.i.c. of 3-Deoxy CAM is low, (10 µg/ml), it nevertheless possesses inhibitory activity indicative of ability to interact with the ribosome. In the MLS system gratuitous inducers have not been found. Pestka et al. (16) in studying a series of erythromycin derivatives, observed that ability to induce and ability to inhibit protein synthesis were inseparable. A key feature in the translational attenuation model for regulation of MLS resistance is the binding of the inducing antibiotic to the ribosome during induction, causing the ribosome to stall at a critical site in the leader sequence of the message.

The observation that the pC194 CAT gene with its promoter retains inducible expression after introduction into E. coli cells suggests further that any other participants in induction might be ordinary cell constituents. The ribosome remains as the only other known cellular constituent capable of binding chloramphenicol specifically. We have examined the 23S rRNA primary structure to see whether a subsequence capable of interacting with the CAT mRNA inverted complementary repeat sequences might be present. The 23S rRNA contains 7 occurrences of the subsequence GGAGG which corresponds to the canonical Shine and Dalgarno sequence (18). One of these occurs in association with the complementary sequence CCUCC, as part of inverted complementary repeat sequence, number 78, described by Noller et al. (14). Examination of this region in 23S rRNA further reveals an exact match between both the CAT mRNA and inverted complementary repeat sequence number 78 extending over the 9 nucleotide sequence 5'CCUCCUAAA, and in both cases pairing between CCUCC and GGAGG is physically possible. We therefore speculate that 23S rRNA contains a part of the information necessary for activation of CAT mRNA.

According to the model that we propose for CAT induction: (i) The rate of CAT mRNA synthesis is unaffected by the presence of the inducer. (ii) The 50S ribosome subunit serves as the macromolecular sensor of CAM for regulation of CAT synthesis. (iii) CAM forms a specific complex with the 50S subunit which alters the conformation of the latter. (iv) As a result of CAM-ribosome complex formation, the paired sequence CCUCCUAAA:UUUAGGAGG at position 2260 of E. coli 23S rRNA becomes functionally accessible to interact with the nascent CCUCCUAAA during CAT mRNA synthesis. (v) The sequence GGAGG

in the nascent CAT mRNA unpaired with the sequence CCUCC in the CAT mRNA would then be free to interact with CCUCC in 16S rRNA with consequent formation of initiation complex with 30S subunits. (vi) Finally, for synthesis of CAT, a 50S subunit not complexed with CAM links up with the initiation complex to form a 70S ribosome active in translation. The 50S subunit thus serves the role of mRNA selection when complexed with CAM and for synthesis of CAT if uncomplexed with CAM.

The initial formation of a complex between the 30S subunit and mRNA followed by a link-up with the 50S subunit is generally accepted as the way in which protein synthesis is initiated (6). In several models of translational regulation, the 30S ribosomal subunit, together with soluble factors and information inherent at the 5' end of mRNA select which message will be preferentially translated [see Kozak (12) for a recent review of these types of mechanisms].

Our current model contrasts with other control mechanisms insofar as it postulates that the 50S subunit makes the initial contact with and selects a particular mRNA for translation. The interaction between 23S rRNA and CAT mRNA need not involve dissociation of the 23S rRNA secondary structure. Experimental evidence for triplex and tetraplex polynucleotide conformations has been reported by Arnott and Bond (1), Arnott and Selsing (2), and Johnson and Morgan (11) make this model feasible.

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PLASMID AND LARVAL TOXIN OF BACILLUS LATEROSPORUS

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Mosquitos are vectors of various diseases which constitute serious health hazards to humans in a number of tropical countries. Microbial control of the mosquito has received attention because of many problems, particularly environmental problems, that have been associated with the extensive use of chemical pesticides.

So far, only 2 species of Bacillus (B. thuringiensis Israeliensis and B. sphaericus) have been reported (1,2) as agents used against mosquito larvae. We recently discovered that B. laterosporus As1.864 isolated from Heilungkiang province in China (Fig. 1-3) exhibited entomocidal activity against different species of mosquito larvae including Culex tritaeniorhynchus (vector of encephalitis) C. pipiens, C. neovishnui, Aedes albopictus, and Anopheles sinensis.

It has been reported that the entomocidal toxin of B. thuringiensis Israeliensis against mosquito larvae is located entirely in the parasporal crystalline inclusion (3), and there is a correlation between the loss of crystal production and the curing of plasmids of 4.0 - 4.4 megadalton (4). The larvicidal toxin activity of B. sphaericus 1593M is present in the cell wall/membrane fraction of presporulating cells (5) and the toxin has not yet been well-characterized. The toxin gene(s) is located on the B. sphaericus chromosome, and a DNA fragment from B. sphaericus coding for biocidal activity against mosquito larvae has been cloned and expressed in Escherichia coli (6). There was no larvicidal activity in vegetative cells of either B. thuringiensis Israeliensis or of B. sphaericus. In contrast, we have found that the larvicidal activity of B.

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Fig. 1. Vegetative cells of Bacillus laterosporus As1.864.

laterosporus As1.864 was reached in exponential phase culture while the bacterial cells were vegetative, and that both bacterial suspension and the filtrates in either exponential- or stationary-phase cultures were effective against the larvae of mosquitos. Therefore, the mosquito larval toxin(s) of B. laterosporus As1.864 seems different from that of B. thuringiensis and B. sphaericus 1593M.

We have examined B. laterosporus As1.864 for presence of plasmid DNA and have found the presence of a large plasmid of 40 megadaltons in size (Fig. 4). Cells of B. laterosporus As1.864 were

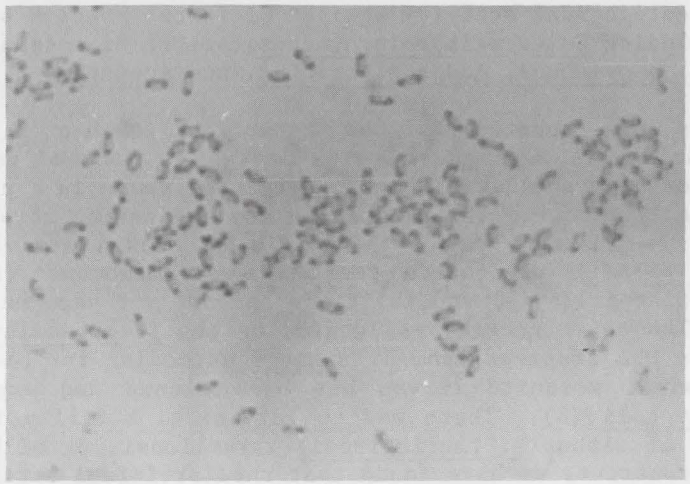

Fig. 2. Photographs of sporangium from Bacillus laterosporus As1.864.

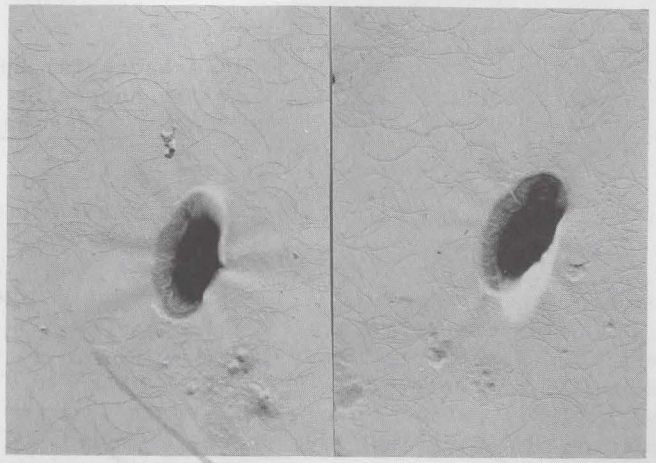

Fig. 3. Electron micrographs of sporangium from Bacillus laterosporus As1.864 (10,000X).

sensitive to antibiotics tested such as gentamicin, erythromycin, ampicillin, tetracycline, chloramphenicol, penicillin, lincomycin, kanamycin, streptomycin, and rifampicin. Establishing the location of the larvicidal toxin gene(s) either on plasmids or on chromosomes is in progress.

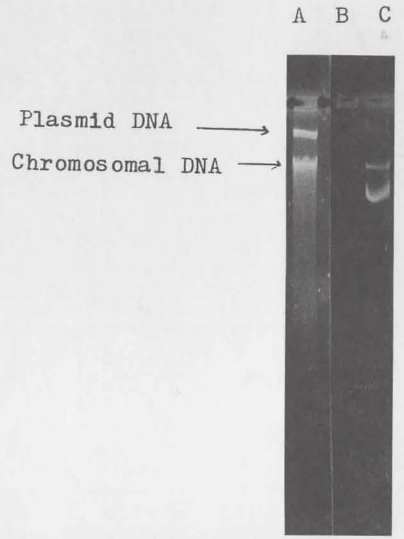

Fig. 4. Agarose gel electrophoresis of Bacillus laterosporus As1.864 plasmid (well A), RPA (well B), and PCM1 (well C).

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THE COMPLETE SEQUENCE OF THE P1 PLASMID MAINTENANCE REGION

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The DNA sequence of a 5-kb region of P1 which encodes sufficient information for stable plasmid maintenance has been determined. This region can be divided into two functionally independent units - one for plasmid replication and the other for partitioning the daughter plasmids to daughter cells.

The unit containing the replication function is 1.5 kb long and includes an origin within a 245-bp segment, the coding sequence for a 32-kd essential protein, RepA, and a 0.3-kb nonessential incompatibility locus, incA. This locus is responsible for the low copy number of the P1 miniplasmid and consists of nine 19-bp repeated sequences. Five similar 19-bp repeats also occur in the origin region (incC). The structural similarities of these two loci suggest that they accomplish their respective functions by related mechanisms.

The partitioning functions are determined by an adjacent 3-kb segment. This region includes the open reading frames for two partitioning proteins, ParA and ParB, and a region, incB, which serves as a "centromere" for partitioning. There are similarities in DNA sequence between the incB locus and the promoter region for parA. These similarities may represent Par protein binding sites that are important for the partitoning process and for autoregulation of the the par operon.

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A MUTANT OF B. SUBTILIS PLASMID pC194 AFFECTING SEGREGATION

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The S. aureus/B. subtilis plasmid pC194 (seg^+) is stably maintained in B. subtilis in the absence of selective pressure. We have characterized a spontaneous plasmid mutant, pC194-1, which segregates rapidly during cell division. Such segregation can be greatly enhanced when cells containing the mutant plasmid are briefly exposed to a pulse of MMS before growth under nonselective conditions.

The mutation causing the seg-phenotype, has been identified as a two base pair addition within the TaqI D+C fragments, extending between bases 2280 and 2790 of the Horinouchi/Weisblum sequence of pC194. The mutation falls within an open reading frame of about 80 bp. Transacting complementation of the defect of pC194-1 could be provided either by restriction fragments from pC194 containing the seg^+ allele or by some unrelated plasmids like pE194.

MMS = Methyl Methanesulfonate

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MASSIVE TRANSCRIPTION OUTSIDE OF THE REPLICATION REGION CAN INTERFERE WITH REPLICATION, MAINTENANCE AND STABILITY OF COL E1 DERIVED PLASMIDS

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Studying structure-function relationships of E.COL 1 expression signals, promoters differing in strength up to seventyfold were investigated in vitro and in vivo. Depending upon their individual strength, the cloning of promoters into plasmids containing the COL E1 or P15A replicon can have a profound effect on copy number, propagation and structural stability of the vector. We therefore, have constructed several plasmid families (1,2), of which the PDS1 system proved to be the most suitable for our purpose. The results of these studies can be summarized as follows:

- overproduction of the ROP-protein reduces the copy number of the plasmid and can contribute to its loss (2).
- readthrough transcription into the replication region from either direction interferes with the plasmids replication and again - depending upon the intensity of transcription - may just reduce the copy number or even prevent propagation of the plasmid.
- all these effects can be overcome by correct placement of strong transcriptional terminators which 'silence' the replication region from outside interference.
- tandemly repeated sequences in transcriptional units outside the replication region can undergo recombination with high frequency if efficiently transcribed.

Based on these findings, the analysis of expression signals in a 'balanced' vector system will be discussed (3).

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ABORTED INSTALLATION OR MAINTENANCE OF MINI-F INDUCES SOS FUNCTIONS

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The SOS inducing potency of UV-damaged F plasmid resides in miniF, a 9.3 kb fragment, coding for the replication and segregation functions of plasmid F. λminiF, a λ carrying miniF, damaged by about 0.5 pyrimidine dimers per kb, induced prophages Ø80 or λ in more than 50% of the infected lysogens. Expression of sfiA, a chromosomal SOS gene under the control of LexA repressor, was also efficiently induced. UV-damaged λminiF, but not the phage λ vector, produced in the host an SOS signal almost as potent as UV-damaged bacterial chromosome, indicating that only miniF is responsible for generating the SOS signal.

We located on miniF a locus, called lynA, 800 bp long, involved in the production of the SOS signal by UV-damaged miniF. We characterized six series of deletion mutants (Rep^-, SOS^-, Fid^-, $Par\text{-}2^-$, $Par\text{-}1^-$, and Par^+) related to four distinct phenotypes displayed by miniF such as partition, copy number, replication, and SOS induction.

We describe a novel mode of SOS induction, called **gratuitous** indirect induction, which is elicited when the maintenance of an intact λminiF introduced into a lysogen was inhibited by a resident plasmid or by mutations in miniF that impaired either its partition or replication. Gratuitous induction required an active RecA protein and a functional miniF $lynA^+$ gene as does UV-indirect induction.

We postulate that impairment of either replication or partition disturbs lynA expression, altering co-segregation of miniF with the chromosome. This entails RecA protein activation through a change in DNA helicity of the DNAs associated with the co-segregation apparatus.

REPLICON FUSION AND THE ORIGIN OF THE INCF GROUP OF PLASMIDS

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IncF plasmids have been divided into subgroups on the basis of their incompatibility behaviour. The cloning of the replication regions of the F plasmid of *E. coli* has demonstrated that more than one region can express incompatibility. Four inc determinants have been identified.

Three basic replicons have been recognized in the IncF1 plasmid group from cloning experiments resulting in the construction of autonomously-replicating miniplasmids. RepF1A is the primary replicon of the F plasmid. RepF1B is the secondary replicon of F and is found in nearly all plasmids of the IncF1 group. RepF1C is a basic replicon showing homology with the RepA region of the incF11 plasmid group (repF11A). Our results show that of the IncF1 plasmids, only R453 carries all three replicons in a functional form. Plasmids of other IncF groups show homology to various extents with these three basic replicons. For example, plasmids R100-1 (IncF11) and R124 (IncFIV) possess an area of homology to repF1B. Representative plasmids of other incompatibility groups have been examined but no homology with the three basic replicons can be detected except for the IncH plasmids which show homology with gene E of repF1A of F.

These results suggest that the subgroups of IncF plasmids may have arisen from an ancestral F-like plasmid which carried only the repF1C replicon and the tra genes. Evolutionary divergence has resulted from various genetic re-arrangements such as replicon fusions, followed by deletion or inactivation of some of the replicons, only remnants of which now remain.

AUTOREGULATION OF THE pSC101 repA GENE

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We have isolated Tn1000 insertions into the replication genes of the pSC101 plasmid, cloned on a colE1 vector. Two of the insertion plasmids can neither transform polA⁻ bacteria nor replicate after a temperature shift in a polAts host. Both transformation and replication activities are restored if the polA⁻ host strains harbor a wild-type pSC101 plasmid. The Tn1000 insertions thus inactivate a transacting replication function repA. The insertions inactivate the synthesis of a 35,000 Mr protein. We have identified the transcription and translation initiation signals of the repA gene. Fusion of these signals to a β-galactosidase gene segment results in strong expression of β-galactosidase activity which is repressed, in trans, by plasmids carrying a cloned repA gene. The repA gene is, therefore, autoregulated. Measurements of transcription from the repA promotor show that this autoregulation operates primarily at the level of transcription. The analysis of repA promotor mutants that are no longer sensitive to autoregulation shows that transcription of the repA gene is repressed by the binding of the repA protein to a segment of DNA, which overlaps the repA promoter.

ELIMINATION OF RESISTANCE PLASMIDS FROM ESCHERICHIA COLI BY 2,2'-DIPYRIDYLAMINE

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Various chemical agents are able to eliminate certain plasmids from their bacterial hosts. Many of these work well against the F plasmid but are not reproducibly effective against resistance plasmids. Using a Tn951 (Lac^+) derivative of the R plasmid R100 (IncF11) we found that 2,2'-dipyridylamine at 500 µg/ml caused reproducible plasmid loss (approximately 8%) from E. coli K12 strain CSH26 after overnight growth in BH1 broth. The resistance markers of R100 were lost simultaneously with the Lac^+ phenotype. Gel electrophoresis of lysates of cured cell lines confirmed loss of the plasmid. Untreated control cultures had <0.5% plasmid loss.

The curing effect of dipyridylamine was not confined to this particular strain, as other K12 strains (C600, JG3272) carrying R100 were also affected. Curing was dose-dependent and occurred at concentrations well below the minimum inhibitory concentration. Curing took place in minimal, as well as rich, medium and was more effective at 30C than at either 37 or 42C. Studies on the kinetics of plasmid elimination indicated that during the exponential phase the number of plasmid-free cells in the population increased at a constant rate. This rate was too rapid to be due solely to the replication of plasmid-free cells, indicating that dipyridylamine acts by interfering with plasmid maintenance, rather than by selectively enriching for pre-existing plasmid-free cells. To determine the plasmid spectrum for curing, one or two representative plasmids of nine incompatibility groups were examined. Dipyridylamine affected the stability of the IncF11 plasmids R1 and R6-5. Stability of the representative of each of the other groups was not affected. Dipyridylamine should be useful in efficiently curing E. coli of certain R plasmids.

PLASMID R1 REPA PROTEIN PROMOTES INITIATION OF R1 REPLICATION THAT IS INDEPENDENT OF TRANSCRIPTION BY HOST RNA POLYMERASE

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RepA is a plasmid R1 coded protein which synthesis is required for replication of this plasmid. A *rep*A recombinant, pS01, was constructed that codes for a fused RepA protein which expression is under the control of the bacteriophage lambda P_L promoter and of translation initiation signals of gene *c*II of lambda. In cells containing pS01, derepression of the lambda P_L promoted resulted in efficient synthesis of the fused RepA protein and in enhanced replication of co-resident R1 miniplasmids. Furthermore, cell free extracts of *E. coli* prepared and assayed as described (Diaz *et al.*, Nature, *289*, 326-328, 1981) promoted efficient synthesis of the fused RepA protein when supplemented with pS01 DNA. Following synthesis and accumulation of RepA in these extracts, semiconservative replication of the R1 miniplasmid pKN182 could be assayed in the absence of further protein synthesis. RepA dependent initiation of R1 replication in this system was found to be independent of transcription by host RNA Polymerase. This was indicated by the insensitivity of pKN182 replication to Rifampicin, as well as to antibodies prepared against whole RNA Polymerase. M13mp8 based R1 recombinants assayed in this replication system, defined the region with which RepA interacts, to the 1 kb *Sau*3A fragment that contains the sequences in which R1 *in vitro* replication starts (Diaz and Staudenbauer, J. Bacteriol. *150*, 1077-1084, 1982).

NEW STABILITY FUNCTIONS OF PLASMID R1 THAT INTERFERE WITH CELL DIVISION ARE CLOSE TO THE ORIGIN OF REPLICATION

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Mutations in the 1.5 kb *Pst*I-*Eco*RI fragment adjacent to the origin of replication of plasmid R1 have been isolated that stabilize the *par*$^-$ miniplasmid pKN1562 without altering its copy number. These mutants slow down the growth of the cell by way of a reversible inhibition of cell division. The stability and interference with cell growth are simultaneously complemented *in trans* by pBR322 recombinants carrying the 1.5 kb *Pst*I-*Eco*RI fragment. A weak incompatibility was found to be specified by this fragment. Selection for fast growth in cells containing the stability mutants leads to the isolation of plasmid derivatives that are slightly more unstable than pKN1562; some of these carry insertions or deletions inside the 3.0 kb *Pst*I fragment that complements the mutations. *In vitro* mutagenesis with hydroxylamine of derivatives with insertions or deletions, give rise to conditional replication mutants of R1 with a frequence of 0.8%. However, the same mutagenesis treatment of the parental miniplasmid pKN1562 gives no replication mutants (frequence < 0.02%). It is proposed that the stability system described here requires the interplay of two diffusible factors and a site and that interactions with the cell membrane, probably at the division plane, are involved. It is also proposed that the role of this system is to prevent the appearance of plasmid free cells that result from failures in replication of the plasmid.

THE REPA2 PROTEIN OF NR1 REPRESSES TRANSCRIPTION OF ONE OF THE MESSENGERS FOR THE REPA1 REPLICATION PROTEIN

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The DNA encoding the repA1 replication protein of the plasmid NR1 is transcribed into two overlapping mRNAs, RNA-A and RNA-CX. Transcription of RNA-CX begins about 350 bp upstream from the 5'-end of RNA-A. The non-overlapping 5'-end of RNA-CX also codes for the protein repA2, which is a repressor of RNA-A transcription. Transcription of RNA-CX is constitutive at a low level and the synthesis of repA2 protein is gene dosage-dependent. This causes RNA-A transcription to vary inversely with plasmid copy number, which is an important component of the NR1 replication control system. The total amount of repA1 mRNA is the sum of the constitutive RNA-CX and the regulated RNA-A.

The lacZ gene was placed under transcriptional control of the RNA-A promoter on a λ prophage. Transcription rates were also measured directly by quantitative hybridization of pulse-labeled RNA. We determined the in vivo relationship between RNA-A promoter activity and repA2 protein concentration by providing a source of trans repA2 repressor which could be regulated by lac control elements. We found that: 1) The repA2-RNA-A promoter interaction is cooperative, probably as a result of active tetramer formation; 2) At the normal copy number of NR1, RNA-A transcription is 96% repressed, and RNA-CX serves as the primary mRNA for the repA1 initiator; 3) Below the normal copy number, RNA-A transcription becomes substantially derepressed, which significantly increases the amount of repA1 mRNA.

SELECTION AND CHARACTERIZATION OF INCOMPATIBILITY MUTANTS OF ColE1 RNAI

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The bacterial plasmid ColE1 has been extensively studies as a model system for the control of DNA replication and plasmid incompatibility. RNAI has been shown previously to be the major control element of ColE1 replication, and is solely responsible for plasmid incompatibility. We have used a rapid selection to generate ColE1 RNAI impaired function mutants. Using the constructed plasmid pTF421Δ,(containing a trp-promoter-ColE1 RNAI gene fusion and Ap^r), mutants in the RNAI gene are unable to segregate away another plasmid containing a ColE1 origin of replication. Mutagenesis of pTF421Δ followed by transformation into cells containing a Cm^r plasmid containing a ColE1 ori, has allowed us to select many mutants in RNAI. These mutants have been analyzed by DNA sequencing, in vitro transcriptions, segregation assays, and other methods. These point mutants are of various classes affecting 1) trp promoter, 2) transcription termination, RNAI loops I and II, 3) RNAI stem I secondary structure, 4) the 5' single stranded tail. These novel mutants allow us to determine the critical structural and functional domains of the ColE1 RNAI molecule.

A NOVEL TYPE OF COPY NUMBER MUTANT OF COL E1

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Copy number control of the plasmid Col E1 has been demonstated by mutational studies to be a function of a 108 nucleotide transcript (RNA1) which, in vitro, inhibits formation of an active primer of replication. RNA1 and primer transcripts converge, primer starting a -555 from the origin and RNA1 at -446. RNA1 transcription has been investigated in vivo for wild-type (cop+) Col E1 and a number of high copy-number mutants by gel analysis of pulse-labeled RNA and by hybridizing RNA blots with RNA1-specific probes. This analysis reveals the 108 nucleotide RNA1 seen in vitro anda shorter RNA (RNA1*) as well. 5' end analysis shows that RNA1* is a processing product of RNA1 from which the first 5-6 nucleotides have been removed. Both RNA1 and RNA1* are very unstable (half-life 1-2 minutes) in vivo despite their extensive secondary structure. Results are similar in cop- mutants. Three of these mutants (cop7, cop3, and cop4) however show an aberrantly stable RNA 1*. RNA1* has a half-life of about 30 minutes in these mutants and accumulates to 5-10% of total RNA. RNA1 on the other hand is no more stable than in cop+-containing cells. The cop7 phenotype is caused by a point mutation between the -10 and -35 regions of the RNA1 promoter. Through studies of plasmid segregation from cells containing both cop+ and cop7 plasmids, I have shown that the cop7 mutant is trans-recessive despite its high copy number and high level of RNA1 transcription.

A SECOND INCOMPATIBILITY FUNCTION ON RP4 INVOLVES PLASMID-HOST INTERACTIONS

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Two plasmid maintenance mechanisms have been shown to be involved in the phenomenon of incompatibility : replication control and partition. The oriV region of plasmid RK2/RP4 determines strong incompatibility (IncP) and appears to contain the replication control system of this plasmid. We have cloned DNA fragments from the trfB/korA/korB region of RP4 into various vectors and noted that some of these also confer an IncP phenotype, which we designate IncPb. The fragment from 54.4 (SstII) to 56.4kb (EcoRI) of RP4 confers IncPb$^+$ whereas the 54.6 to 56.4kb region is inactive. Both these fragments express the korA$^+$ gene product but the latter was obtained from an RP4::Tn76 derivative which is unstable, reduces its host's growth rate and has suffered a reduction in host-range.

IncPb differs from incompatibility mediated by a replication control region in that dual replicons do not suffer loss when challenged by either replicon, whereas our IncPb$^+$ clones do. There is no evidence that the IncPb$^+$ fragment can act as a replicon, neither does it stabilize pACYC184 ie it is not a par region. We have noted, however, significant effects of IncPb$^+$ clones on the growth rates of their hosts especially when in a dual plasmid incompatibility situation. We suggest that the IncPb phenomenon is the result of plasmid-coded products interacting with host growth or replication mechanisms. The intact IncP plasmid normally provides further interacting products which relieve the host and provides a plasmid maintenance mechanism active across its broad host-range.

A RECOMBINANT PLASMID WITH INCREASED COPY NUMBER IN BACILLUS SUBTILIS Ki-2-132

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Since cloning system of B.subtilis has special advantages, it becomes an important system for cloning and expression of foreign genes. Ehrlich, Dubnau and Lovett et al have introduced Staphylococcus aureus plasmid pUB110, pCM194, pSA2100 pE194 into B.subtilis. Among them pUB110 has been used most extensively, because its copy number and transformation efficiency is higher than others, but it is still lower than some plasmids of E.coli. It is important for cloning system of B.subtilis to reconstruct plasmid vector having high copy and transformation efficiency. This paper reported recombinant pK209-8 constructed with plasmid pUB110 digested with restriction endonuclease BamHI and EcoRI and BamHI-EcoRI fragment of chromosomal DNA of B.subtilis 209. The copy number and transformation efficiency of the reconstructed plasmid both are higher than pUB110 and the molecular weight is $4.5X10^6$ daltons. Its copy number is 101 per cell, 1.9 times that of pUB110, with transformation efficiency 10 times higher than that of pUB110.

CHARACTERIZATION OF THE REPLICATION AND STABILITY REGIONS OF THE *AGROBACTERIUM* PLASMID pTAR

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The 44 kb *Agrobacterium* plasmid pTAR, from the non-oncogenic grapevine strain 1D1422, confers on its host the ability to catabolize tartrate and is compatible with the Ti plasmids pTiC58 and pTiAch5. We have mapped the origin of replication and the partition locus in a 5.4 kb region of the plasmid. Within this region is a 1.3 kb segment that is capable of directing autonomous replication. The remaining segment contains the partition functions necessary for stable plasmid inheritance of pTAR during nonselective growth. Using a cell-free coupled transcription-translation *Agrobacterium* system, we determined that at least two proteins, 7.8 kd and 20 kd and possibly a third protein, 26 kd, are encoded by genes in a tightly clustered arrangement which defines the region involved in partitioning. Deletions within this region render the plasmid unstable with the concurrent loss of these proteins, suggesting that one or more of these proteins may be involved in efficient partitioning of pTAR.

PARTITIONING OF PLASMID R1: TWO DIFFERENT LOCI REQUIRED FOR COMPLETE STABILITY

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The low copy number plasmid R1 has been characterized very well with respect to replication control functions, whereas the analysis of the *par* genes has only just started. We present data showing, that R1 codes for two independent stability regions.

It was previously indicated, that the largest EcoRI fragment (EcoRI A) of R1 is involved in partitioning. This was verified by cloning the EcoRI A-fragment on a *par*⁻ miniR1 replicon: The recombinant plasmid was completely stably inherited. By a series of subclonings it was shown, that the EcoRI A-fragment carries two stability regions, which we designated $parA^+$ and $parB^+$. Each region stabilize R1 partially; $parA^+$ and $parB^+$ replicons are typically lost with a frequency of 10^{-4}/cell/generation, whereas miniR1 replicons containing no *par* region are lost with a frequency of 10^{-2}/cell/generation. MiniR1 replicons containing both *par* regions have loss frequencies in the range of 10^{-6}/cell/generation.

Both par regions exert allel-specific incompatibility (e.i. $parA^+$ plasmids dislodge $parA^+$ stabilized plasmids, and $parB^+$ stabilized plasmids dislodge $parB^+$ stabilized plasmids). No cross-incompatibility between $parA^+$ plasmids and $parB^+$ plasmids has been detected, whereas $parB^+$ plasmids show weak incompatibility towards $parA^+$ $parB^+$ stabilized plasmids.

The *par* regions were cloned on different pl5 plasmid vectors, and it was shown, that $parB^+$ will confer a high degree of stability on these replicons. In contrast, the $parA^+$ region has no effect on the stability of such replicons. Whether the $parA^+$ region is able to stabilize other low copy number plasmid awaits further experimentation.

The two *par* regions were mapped more precisely using exonuclease Bal31. The $parA^+$ region is confined to a DNA fragment of 1500 bp near one end of the EcoRI A-fragment. The nucleotide sequence of the *parA* region confirmed the presence of a 36 K protein detected by the maxicell method. By constructing transcriptional- and translational gene fusions between this gene and the *lacZ* gene, it was shown, that the *parA* gene is autogenously regulated.

The $parB^+$ region, located at the other end of the EcoRI A-fragment, resides within 575 bp and also in this case, the entire nucleotide sequence was determined. The region contains two open reading frames, corresponding to two very small polypeptides. In addition, regions with symmetric sequences are present.

The mechanisms by which the two *par* regions act are unknown, but we have indications, that the $parB^+$ region couples plasmid replication to the host cell cycle.

PLASMID STABILITY: INVOLVEMENT OF A PLASMID-CODED PRIMASE AND SITE-SPECIFIC RECOMBINATION/RESOLUTION SYSTEMS

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Plasmid RP4 in E.coli is found predominately as monomeric DNA molecules with a copy number of 4-5 per chromosome equivalent. In this state it is stably inherited. Deletion derivatives were isolated which show high levels of instability and which form multimers by recA-dependent recombination. Complementation studies demonstrate that RP4 multimerisation is normally prevented by at least two distinct mechanisms: one involves the plasmid-coded primase. A cloned 12kb primase-encoding fragment from the Tral region of RP4 also suppresses a temperature-sensitive mutation affecting the E.coli single-stranded DNA-binding protein; this activity may limit recombination between RP4 monomers. The second mechanism acts by efficiently resolving any plasmid multimers to monomers via a site in the 28-34kb region. Inactivation of primase or deletion of the resolution site leads to low levels of plasmid instability (0.2 or 0.9% loss per generation, respectively); elimination of both primase and the resolution site causes a high rate of plasmid loss (5-7% per generation) and an accumulation of plasmid multimers. Spontaneous revertants with partially restored stability show reduced multimerisation and result from deletions or insertions close to the RP4 origin of replication. We propose that this region contains a hot spot for homologous recombination. Multimerisation probably disrupts normal partitioning and the results are consistent with only limited numbers of RP4 molecules being actively segregated at cell division.

PLASMID RSF1010 DNA REPLICATION IN VITRO PROMOTED BY PURIFIED RSF1010-ENCODED repA, repB and repC PROTEINS

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A soluble enzyme system for the study of plasmid RSF1010 DNA replication has been developed. The system consists of a partially purified mixture of Escherichia coli replication proteins (fraction AS[0-73]) and three highly purified plasmid-encoded proteins, the products of the RSF1010 genes repA, repB and repC. DNA synthesis in this system closely resembles the known characteristics of RSF1010 replication that occurs in vivo or in vitro with crude extracts of RSF1010-harboring cells. The system (i) depends completely on externally added DNA, (ii) replicates specifically supercoiled plasmid DNA or phage replicative form DNA containing the RSF1010 replication origin (oriV), (iii) depends on each of the three RSF1010-encoded rep proteins, (iv) depends on host replication proteins such as DNA gyrase and dnaZ protein but not on others (e.g. RNA polymerase, primase, dnaB protein and dnaC protein), as indicated by specific antibiotic or antibody inhibitions or by the activity of enzyme fractions derived from dna(ts) mutants of E.coli, and (v) creates replication 'bubbles' at or near oriV which are extended either bi- or unidirectionally to generate two complete supercoiled product molecules. Provided that a unique RSF1010 origin DNA sequence is present, circular single-stranded templates are also active in this in vitro system. In contrast to the observations with duplex DNA, the ssDNA-dependent initiation reaction requires only one of the three RSF1010-encoded rep proteins: the repB protein.

REPLICATION KINETICS OF THE *STAPHYLOCOCCUS AUREUS* PLASMID pT181

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pT181 is a 4.4 kb., 20-25 copy, *S. aureus* Tc^r plasmid whose replication rate is determined by the concentration of a *trans*-active initiator protein, RepC. RepC synthesis is negatively regulated by two small RNA countertranscripts that are read from the opposite strand of the 5' end of the RepC mRNA leader. A clone that produces these small RNAs inhibits pT181 replication *in vivo*. Complementary GC to AT base changes in the interacting regions of the inhibitor and mRNA leader lower the efficiency of the inhibitory interaction and result in increased plasmid copy number. Deletion mutations within this region abolish the negative interaction and produce copy mutants with even higher copy numbers.

The kinetics of pT181 repopulation were studied using thermosensitive replication mutants (*tsr*) of the wild-type plasmid and a copy mutant having a GC to TA point mutation in the inhibitor-target region. Strains were grown in a turbidostat at 42° to lower plasmid copy number and were then shifted to 32°. Plasmid replication and copy number were followed after the downshift. Both the wild-type and the copy number mutant plasmid exhibited an initial exponential replication phase resulting in a peak copy number that was 2-3 times higher than the steady-state value. This overshoot was followed by a prolonged period of damped oscillation about the steady-state level.

Plasmid repopulation kinetics were also examined following introduction of up to six plasmid copies per naive cell by infection with the high frequency transducing phage, ϕ11. A wild-type pT181 replicon exhibited overshoot kinetics similar to those seen for the *tsr* wild-type plasmid, while a copy number mutant of pT181, having a small deletion in the inhibitor-target region, did not overshoot its steady-state copy number, but instead repopulated by hyperbolic kinetics.

The overshoot results are interpreted as being due to asynchronous cycles of RepC and inhibitor synthesis; the protein accumulates until its synthesis is inhibited by the accumulation of repressor. The overshoot and damped oscillation shown by a pT181 point mutant are consistent with its retention of inhibitor function. The hyperbolic kinetics shown by the deletion mutant indicate that this plasmid is no longer subject to negative replication control by the RNA species.

RELATIONSHIP BETWEEN PLASMID COPY NUMBER AND MOLECULAR SIZE OF THE PLASMIDS COLE1 AND NR1

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Derivatives of the unrelated plasmids ColE1 and NR1 had a similar relationship between plasmid copy number and molecular size. ColE1 derivatives 2 to 12 kb in size had a constant copy number of 16.5 as determined by dot-blot hybridization of cesium chloride-ethidium bromide density gradient fractions. The copy numbers of ColE1 derivatives larger than 12 kb decreased as their molecular size increased. The larger ColE1 derivatives were maintained at a plasmid mass equal to 6 percent of the total cellular DNA. The location of the DNA insertions and deletions did not effect this relationship. The copy number of NR1 was constant for molecular sizes ranging from 5 to 95 kb. A point mutation in the small repressor RNA of NR1 resulted in a dependence of plasmid copy number on molecular size. Derivatives of the mutant, pRR12, were maintained at 8 percent of the total cellular DNA for the entire size range studied (5 to 95 kb). The difference between the NR1 and pRR12 relationship is identical to that found for ColE1 and its copy mutant, pOP1 (Muesing, M. and B.Polisky, p. 412 In D. Schlessinger (ed.), Microbiology-1981, ASM). pOP1 also has a single base pair alteration in its repressor RNA molecule.

These data suggest intracellular limitations of the host cell become involved in copy number control when the total plasmid mass reaches a critical level. The wild-type plasmids of NR1 and ColE1 are maintained at mass levels below this critical point. The molecular size of ColE1 had to be doubled before its copy number was size dependent. Since runaway replication mutants exist, plasmid mass is not the only factor controlling the copy number of pRR12, pOP1, and the larger ColE1 derivatives. More likely there is a balanced interaction between a limiting intracellular factor(s) and the activity of the repressor of plasmid replication.

STABILITY OF THE PLASMID COLE1 IN ESCHERICHIA COLI

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ColE1 plasmids encoding a functional bacteriocin gene were stable in growing populations of E. coli. These cells excreted colicin into the medium which inhibited the growth of colicin sensitive cells. ColE1 plasmids encoding non-functional colicin genes were unstable and did not release the inhibitory activity. The stable ColE1 plasmids became unstable in recA and tolC bacterial strains. Colicin expression is repressed in recA strains. The tolC gene specifies a membrane protein which renders the strain resistant to the killing effects of colicin when mutated. The results suggest the low level expression of uninduced colicin killed the cured cells in the growing populations and caused the plasmid ColE1 to have a stable phenotype.

ColE1 was a comparatively stable plasmid even with a non-functional colicin gene. The number of plasmid segregating units was responsible for the relative stability of ColE1. Two factors determined the number of plasmid segregating units present in the cell. They were the average copy number of ColE1 (16.5) and the ability of ColE1 to resolve into monomeric forms after replication. The high number of plasmid segregating units caused a very low frequency of cured cells. Also important for the stability of the ColE1 plasmid was the difference in growth rate of cells with and without ColE1 plasmids. The differential growth rate caused by ColE1 was orders of magnitude greater than the rate of cured cell formation. Consequently differential growth rate dictated the downward concavity of the stability curve. The number of plasmid segregating units influenced the number of generations before the loss of the plasmid was initially observed.

Considering the number of plasmid segregating units in the cell and the differential growth rate in the cell population, ColE1 was calculated to be segregated to daughter cells by a mode of random binomial distribution. This would mean ColE1 lacks a par locus. Therefore, a high number of plasmid segregating units and the low level expression of its bacteriocin are adequate to stabilize the plasmid ColE1 in an exponential population of E. coli cells.

A PROPOSED MODEL FOR CONTROL OF MINI-F REPLICATION

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Mini-F requires an origin gene and the repE plasmid gene, which encodes a 29 kilodalton (kd) protein, to form a replicon. Two copy number control genes have been identified; they are copA and copB. Here we report that copA mutations, all of which map in repE, inactivate autogenous regulation of the repE promoter. This indicates that the 29 kd protein is an autorepressor as well as essential for replication. In order for the 29 kd protein to fulfill its indicated roles, we propose: (1) that in a simple form this protein acts as an autorepressor thereby establishing its steady state concentration; and (2) that at a rate dependent on this concentration, a small fraction of autorepressor is modified to function as an initiator. Since the copB DNA sequence mimics the origin sequence, we also propose that copB competitively binds initiator thereby further reducing its availability.

A MINIMAL REGION OF AN IncN-GROUP PLASMID REQUIRED FOR ITS MAINTENANCE IN ESCHERICHIA COLI

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The 39 kb plasmid pCU1 is a broad host range N group plasmid that is being studied in some detail. In addition to phenotypes characteristic of the group, the plasmid also confers resistance to ampicillin, streptomycin and spectinomycin. Electron microscopy of denatured molecules of pCU1 revealed four different stem-loop structures. Their dimensions, positions with respect to one another and a restriction and functional map of the plasmid have been described previously. Of particular interest was the assignment of the maintenance region of the plasmid to a location within one of the stem-loop structures.

The deletions used to locate various phenotypes of pCU1 collectively encompass all but a continuous 6 kb region of the plasmid. This and the ability of the smallest deletion derivative, pCU32, to be stably replicated and maintained in E. coli indicated that ampicillin-resistance (the marker on pCU32) and all of the plasmid-specified replication functions are within this 6 kb of DNA. To locate the maintenance region within pCU32, Tn5 insertions of this plasmid were isolated. Their distribution defined: (a) an Ampicillin-resistance region, (b) a region for stable maintenance, (c) a region insertions into which had no effect on pCU32, and (d) a region insertions into which were associated with deletions extending into and removing c. c could not be cloned in the absence of d suggesting that they may contain genes specifying functions analogous to the kil and kor (kill override) functions that have been found to be specified by plasmid RK2.

Regions a, c and d occupy about half of pCU32. The minimal maintenance region inferred to be on the other half was defined by Tn5 insertions delimiting a 2 kb region. Most of this region was present on one of five PvuII fragments of pCU32. Replication of pCU32 was polA-independent. Each PvuII fragment of pCU32 was cloned into the single PvuII site of the polA-dependent plasmid pBR329. Subsequent removal of the replication region of pBR329 and selection of plasmids in an E. coli polA mutant resulted in the isolation of plasmids that always contained PvuII fragment 2 of pCU32. Most of the maintenance region that was defined by the distribution of Tn5 insertions is within this PvuII-2 fragment. To further demonstrate that the PvuII-2 fragment contains all that is necessary for stable plasmid maintenance, PvuII-2 was purified and linked in vitro to the non-replicating tetracycline-resistance fragment of plasmid pMK16. Such constructed plasmids were stably maintained without antibiotic selection in wild type and in polA strains. We conclude that all plasmid-specified functions and sites needed for the stable maintenance of this prototype IncN group plasmid are determined by this single region. We will further dissect and sequence this region.

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REGIONS AROUND THE E. COLI ORIGIN OF REPLICATION WHICH AFFECT THE STABILITY OF PLASMIDS

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Joint replicons were constructed which contain a minichromosome - a plasmid replicating from the E. coli chromosomal origin of replication - and miniF or pSC101, respectively. oriC::miniF cointegrates were completely stable, in agreement with published results (Ogura, T. et al., PNAS 77, 3993-3997, 1980). Hybrids between pSC101 and minichromosomes pOC3 (9.5 kb EcoRI fragment, coordinates -5.8 kb to +3.7 kb of the oriC sequence) and pOC15 (6.0 kb HindIII-EcoRI fragment, coordinates -2.3 kb to 3.7 kb, Messer et al., Cold Spring Harbor Symp. Quant. Biol. 43, 139-145, 1978) were unstable: 40-60 % of the cells were free of plasmid when grown for about 15 generations without selection. This demonstrates that not only is the partition function of pSC101 unable to stabilize these minichromosomes (Hinchcliffe, E. et al., Plasmid 9, 286-296, 1983), but in addition, the presence of sequences around oriC cause unstable partition of pSC101. This effect was only observed in cis.

Surprisingly the region close to and including oriC and suggested regulatory elements like the 16 kd and 17 kd proteins with putative oriC specific incompatibility regions did not interfere with pSC101 stability. Cointegrates containing the oriC-HincII fragment (coordinates -0.18 kb to +1.5 kb) were stable.

Thus, oriC replication properties as well as the sequence homology in the 17 kd protein with the par function of pSC101 (Miller, C. A. et al., Gene 24, 309-315, 1983) are not responsible for the instability imposed on pSC101. The destabilizing sequence is distant from oriC.

INFLUENCE OF REGIONS UPSTREAM THE PROMOTER FOR THE PRIMER RNA ON THE COPY NUMBER AND STABILITY OF pBR327 - DERIVED PLASMIDS

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Replication of plasmid pBR327 and its derivatives proceeds by a ColE1 - type mechanism whose key elements are two RNA molecules (RNA II, and which serves as primer; RNA I, which acts as inhibitor) and the rop gene product (Som et al. PNAS 80: 3232 (1983).

Using recombinant DNA methodologies we have constructed a series of plasmids, derived from pBR327, which altered copy number and stability phenotypes.

One derivative resulted from a deletion that removes from position 2037 to position 2956 of pBR327 (Soberón et al., 1980) (from -43 and up the primer promoter) and eliminates two transcriptional terminators.

This plasmid has an increased copy number, probably due to transcription originating upstream the origin region. Insertion of a small (12 p. b.) DNA segment at position -43 of the primer promoter in this plasmid caused a decrease in copy number to below the levels of the parental plasmid pBR327. Another deletion that removes the most proximal of the upstream promoters also causes a decrease in the copy number. These and other results suggest that the region immediately upstream the primer promoter is important for the replication mechanism.

Our results allow us to draw the following conclusions. They confirm that transcription coming from upstream the RNA II promoter can generate primers and, this increases the copy number. Since the region comprised between -40 and -55 cannot be affected without and effect in copy number, the region may be involved in some kind of interaction with regulatory molecules.

The derivatives of pBR322 studied lack a mechanism of stability independent of copy number; its segregation appears to depend solely on the latter.

FUNCTIONAL LIMITATIONS OF THE par LOCUS OF pSC101 AND FURTHER CHARACTERIZATION OF ITS PHENOTYPES

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The par region of pSC101 has been sequenced (Miller et al., 1983, Gene 24: 309-315) and deletion analysis has been done to determine the functional par locus (Tucker, Miller and Cohen, Cell, 1984). Within a 100 bp sequence there are three discrete PR (partition-related) segments that contribute incrementally to expression of partition.

Deletion of any two segments within the par region has been correlated with the Par$^-$ phenotype. Deletion of the entire region leads to more rapid loss of the plasmid than would be predicted from a model involving random distribution of a plasmid pool of the size known to exist for pSC101. Deletions that extend into either of the lateral PR segments, but leave the internal segment and the other lateral segment intact, are stable (i.e. are Par$^+$); however, these show a phenotype described as Cmp$^-$. A Cmp$^-$ plasmid is always lost preferentially when competing with a co-existing incompatible wild-type replicon (hence the name Cmp).

AUTOREGULATION OF THE F FACTOR PIF OPERON

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The F factor pif operon comprises at least three structural genes, pifC, pifA and pifB, located near the primary origin of mini-F replication (oriV1, F coordinate 42.6kb). The pifA and pifB gene products are responsible for abortive infection of bacteriophage T7. We have studied the regulation of pif expression using protein fusions of pifC, A and B to lacZ. The product of pifC negatively controls its own expression as well as expression of pifA and pifB. A lacZ operon fusion to pifA is repressed by the pifC product, suggesting that pif regulation is at the level of transcription. A Tn5 insertion near oriV1 is polar on pifC, pifA and pifB, showing that these genes are co-transcribed.

We have identified an additional element, pifO, which is the site required in cis for autoregulation of pif expression by the pifC product. Spontaneous mutations in pifO (pifO36, pifO55, pifO70) result in increased expression of pifC-lacZ product in the presence of pifC in trans. Multiple copies of pifO (mapping within 42.9kb-42.43kb) in trans to F'lac result in a 140-fold increase in T7 inhibition. This is due to titration of pifC product by pifO sequences in trans and concomitant expression of pifA and pifB. pifO36, pifO55 and pifO70 sequences have little effect on F'lac pif expression in trans.

Tanimoto and Iino (Mol. Gen. Genet. (1983)192:104-109) have shown that the F factor inhibits transfer of the broad host range plasmid RP4. The gene responsible was localized to the region we assign to pifC. Our recent results show that RP4 titrates pifC product in trans to F'lac and that pifC is responsible for F factor inhibition of RP4 conjugal transfer.

IDENTIFICATION AND ANALYSIS OF REPLICATIVE INTERMEDIATES OF E. COLI ORIC PLASMIDS DURING IN VIVO AND IN VITRO REPLICATION

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Replication of E. coli minichromosomes was investigated in vitro and in vivo to identify and analyze replication intermediates. In vitro replication was as described by Fuller et al. (Proc. Natl. Acad. Sci. 78, 7370, 1981). In vivo replication was analyzed in temperature-sensitive dnaA mutants incubated at various temperatures. Replicative intermediates in both systems were labeled with tritium in pulse-labeled and pulse-chase experiments, and examined by agarose gel electrophoresis and fluorography. The major intermediates detected were strikingly similar in vivo and in vitro. Minichromosomes replicated rapidly to an OC-OC catenated dimer form, followed by slower progression to CC-OC and CC-CC dimers and finally to CC supercoiled monomers, with ladders of forms which migrated to intermediate positions in the gels. This processing through the dimer forms required several minutes at 25-30°C in both systems. The slow manner in which one form progressed to another compared to the much shorter time necessary to polymerize the nucleotides into DNA, adds further support to the notion that this processing is rate-limiting in minichromosome replication. Further, the similarity of the in vivo and in vitro events demonstrates the fidelity of the in vitro replication system. However, there were differences in the complexity and quantity of some intermediates when different minichromosomes were replicated. Among the most obvious differences was the very early formation of large, open circular multimers in some minichromosomes and not in others, or at very reduced levels. This may provide evidence for initiation of replication on the supercoiled CC-CC dimer of some minichromosome species.

COLE1 PLASMID REPLICATION IN AMBER RNH (RNASE H) MUTANTS OF ESCHERICHIA COLI

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We have previously reported a system for isolating ColE1 mutants defective in the initiation of DNA replication in E. coli (Naito & Uchida, PNAS 77, 6744 (1980)). These mutations were named cer, signifying a defect in ColE1 replication. One of these mutations, cer6, was a single base-pair alteration 160 base-pairs upstream of the ColE1 replication origin. We then proceeded to isolate and characterize E. coli suppressor mutants of cer (Naito, et al., PNAS 81, 550 (1984)). These suppressors were named her for host factor affecting ColE1 replication. One of the cer6 suppressors (herA) was genetically located within the rnh (RNase H) gene. This alteration correlated with the loss of RNase H activity, leading to the suggestion that replication of cer6 DNA in vivo is sensitive to RNase H activity. If this inference is correct, it should be possible to isolate amber mutations in the rnh gene by selecting for bacterial mutants capable of suppressing replication of cer6 DNA under supO conditions only. Two such bacterial mutants were obtained. By DNA sequencing, both were found to bear amber mutation in the rnh gene. In these mutants, the wild type ColE1 replicon replicated normally. Therefore, we conclude that RNase H activity is dispensable for replication of ColE1 DNA as well as for the E. coli chromosome. This is contrary to the in vitro results which show that RNase H activity is essential for replication of ColE1 DNA (Itoh & Tomizawa, 1980).

Another suppressor of the cer6 mutation (herB) was isolated, and was found to be closely linked to the polA gene on the genetic map of E. coli.

PT181 REPLICATION IS REGULATED INDIRECTLY BY TWO COUNTERTRANSCRIPTS

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The replication rate of pT181 is controlled by an inhibitor-target mechanism similar to that of R1. The target consists of two tandemly initiated leader sequences for the mRNA of a plasmid replication protein, RepC. The inhibitor consists of two countertranscripts complementary to the RepC mRNA leader. These have a common start within the leader region and tandem termination sites about 85 (RNA I) and 140 (RNA II) bp upstream. Most mutations causing increased copy numbers are located in the leader-countertranscript overlap region and involve a GC to AT change in a 12 nucleotide GC-rich segment in that region. Mutations affecting termination of either RNA I or RNA II or their common promoter also cause increases in copy number. In computer-predicted secondary structures of the leader region, the 12-nt target segment forms a single-stranded loop at the end of a hairpin early during transcription of the leader; later, the leader is predicted to re-fold into a new configuration in which the 12-nt segment is largely base-paired. It is likely that complementary base pairing between inhibitor and target RNAs, initiated early during leader transcription, facilitates a secondary folding of the downstream part of the leader that occludes the repC mRNA ribosome binding site within a stem-loop. A deletion that eliminates the countertranscript promoter result in a 15-fold increase in copy number; if the segment that can pair with the ribosome binding site is also deleted, the copy number increases by 50-fold, suggesting that (in the absence of the countertranscripts), the mRNA transcripts are utilized 3x more efficiently if the ribosome binding site is unable to participate in a stable secondary structure. A mutation that decreases the stability of the secondary structure involving the ribosome binding site causes a 20-fold increase in copy number even in the presence of excess RNA I and RNA II.

Because RepC is fully trans-active, pT181 and its copy mutants behave differently in cis-trans incompatibility tests than do those of the directly regulated ColE1 or IncFII plasmids. Although the cloned pT181 inhibitor gene blocks replication of the WT plasmid and inhibitor-sensitive mutants, dominant copy mutants do not exclude either in the heteroplasmid state, nor do mutations that change the specificity of the inhibitor-target interaction give rise to new incompatibility types. Because all mutants share a common RepC pool, all combinations show symmetric segregational incompatibility with segregation rates that are predictable from copy numbers.

THE P1-PAR REGION CAN PARTIALLY STABILIZE OTHER REPLICONS LACKING A PARTITION SYSTEM

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A 3Kb region of P1 [the *par* region] essential for the stable inheritance of the P1 plasmid can also ensure the stable inheritance of F when joined to a *par*$^-$ F replicon. [Austin and Abeles (1983) J. Mol. Biol. 169, 353.] In order to test whether P1 *par* is able to promote the stable inheritance of other unstable replicons we cloned the *par* fragment of P1 [as defined by Austin and Abeles] into other unstable plasmids. These include the *oriC* plasmids pEH21 and pEH22 [Hinchliffe *et al.*, (1983) Plasmid 9, 286.], pPM22, a *par*$^-$ plasmid derived from pSC101 [Meacock and Cohen, (1980) Cell, 20, 529.] and pBR325. The *oriC* and pPM22 derived plasmids were transformed into a *recA* host. The plasmids derived from pBR325 were transformed into WT286, a strain presumed to be partially deficient in *polA* activity because *polA* dependent replicons have a reduced copy no. [4-6]. The stability of the plasmids in these strains was then examined.

pPM22 was completely stabilized by the presence of P1-*par*. The pBR325 and *oriC* P1-*par* hybrids were more stable than their *par*$^-$ parents but were not fully stabilized. The stabilization observed could not be attributed to improved replication as the copy number of the par$^+$ derivatives was not increased. We attribute the inability of the P1-*par* region to fully stabilize the *oriC* plasmids and pBR325/WT286 to the fact that these plasmids probably fail to replicate with complete regularity.

FUNCTIONING OF A TRANSCRIPTION TERMINATOR STRUCTURE IS DEPENDENT ON METHYLATION: ROLE OF METHYLATION IN CLO DF13 PLASMID REPLICATION

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Plasmid Clo DF13 (M.W. 6 Md) contains a relative small DNA region, located between 1.8 and 11.5%, essential for autonomous replication. Several replication mutants of Clo DF13 have been isolated and characterized. One of these mutants, Clo DF13 cop-1 (TS), has a temperature-dependent plasmid copy number and shows a "runaway" behaviour. We have determined the nature of this mutation, located within a region involved in the initiation of RNA II and the termination of the cloacin operon transcript. Our data show that the mutation results in a pleiiotropic effect, namely a non-conditional increase in promotor activity and a temperature dependent decrease in termination efficiency. Recently, we obtained evidence that the absence of dam-methylation in the cloacin terminator structure affects plasmid replication and leads to a loss of the cop-1 (TS) phenotype. In order to study the molecular basis of this phenomenon, we determined the efficiency of termination of the region harbouring the cop-1 (TS) terminator structure in the presence and absence of dam methylation. The data obtained indicate that the absence of methylation results in an increased termination efficiency.

HOST RANGE REPLICATION AND MAINTENANCE DETERMINANTS OF IncP GROUP PLASMID RK2

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Extensive deletion analysis of the broad host range IncP plasmid RK2 has identified two regions of the 56 kb RK2 replicon, a 700 bp origin of replication (oriV) and a 1.8 kb region designated trfA*, which together encode stable replication in Escherichia coli and in the distantly related species Pseudomonas putida (1, 2, 3). Utilizing the RK2 origin of transfer (oriT), the trfA* and oriV segments and larger RK2 derivatives were introduced into and maintenance properties assessed in a variety of Gram-negative species. A trfA*, oriV replicon encodes RK2 replication functions in the nine hosts studied yet maintenance is stable (less than 0.2% loss per generation) in only four of the nine; Escherichia coli, Pseudomonas putida, Pseudomonas aeruginosa, and Azotobacter vinelandii. Stable RK2 maintenance in the other five species studied is dependent on two regions of RK2 designated host range maintenance A (hrmA) and B (hrmB). The hrmA and hrmB regions map at RK2 coordinates 16.0 to 20.0 and 52.6 to 55.7, respectively. HrmA stabilizes mini-RK2 maintenance in Acinetobacter calcoaceticus while the hrmB locus stabilizes mini-RK2 replicons in Rhizobium meliloti, Argobacterium tumefaciens, Rhodopseudomonas sphaeroides,and Caulobacter crecentus.

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REPLICATION DETERMINANTS OF THE BROAD HOST RANGE PLASMID RSF1010

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RSF1010 is a small (8.7kb) multicopy plasmid of the IncQ group that has an exceptionally broad host range among Gram-negative bacteria. This latter property has made it attractive for the construction of DNA cloning vehicles that may be used outside enterics and has motivated studies on its mode of replication.

Cloning of specific regions of RSF1010 has shown that a 2.1 kb segment of the plasmid that contains the replication origin (oriV) can replicate in E.coli if another DNA region, that is located remote from oriV, is present in the same cell on an independent plasmid. Deletion analysis of the replication region has revealed three genes, repA, repB and repC, each being essential for replication of the plasmid in E.coli. The rep genes have been cloned into plasmid ptac12 such that their expression was controlled by the tac promoter. Inactivation of the lac repressor by IPTG resulted in an overproduction of the repA, repB and repC proteins until they constituted 4 to 20% of the total cellular protein. Direction of transcription of each rep gene, as deduced from the cloning experiments, is clockwise on the standard map of RSF1010. This was confirmed by nucleotide sequence determination of the rep region.

Our finding that RSF1010, in spite of its small size, has maintained in the evolution a set of at least three replication genes suggests that these genes make the plasmid independent of certain host determined replication functions and thus allow the plasmid to be replicated in a wide range of different bacterial species.

INTERACTION IN VITRO BETWEEN RNA MOLECULES INVOLVED IN COLE1 DNA REPLICATION CONTROL

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The plasmid ColE1 encodes a small RNA molecule (RNA1) which is a repressor of plasmid DNA replication. This repression involves an interaction between RNA1 and the replication primer RNA. Previous studies have shown that RNA1 consists of three stem/loop structures and a single-stranded 5'end. Thus far,only the loops have been implicated in the functioning of the molecule.

We have developed an in vitro system which allows detection of the complex formed when RNA1 and primer interact. Using this system,we have studied the ability of wild-type and mutant RNA1 molecules to interact with primer RNA. By testing RNA1 molecules with point mutations or small deletions at the 5' end,we have shown that,in addition to the loops,the 5' single-stranded region of RNA1 is also critical to its function. This system has also been used to study the physical parameters (Tm and nuclease susceptibility) of the complex formed by wild-type RNA1 and primer. The data suggest that the end product of the interaction is an RNA:RNA hybrid involving all of RNA1 and the 5' end of primer.

REGULATION OF REPLICATION AND MAINTENANCE FUNCTIONS OF BROAD HOST RANGE PLASMID RK2

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Plasmid RK2 is capable of conjugal transfer between and stable maintenance in most Gram negative bacterial species. The locations of plasmid genes are given by reference to kb coordinates clockwise from the unique EcoRI site on the recently recalibrated 60 kb circular genome. For replication RK2 encodes $oriV_{RK2}$ (12.3 to 13.0 kb), the origin from which vegetative replication proceeds unidirectionally anticlockwise, and trfA (16.0 to 17.3 kb) which encodes a polypeptide(s) essential for $oriV_{RK2}$ function. In addition to these functions various kil loci which have host or plasmid deleterious effects and whose expression is controlled by kor loci have previously been mapped. By analogy with various genes recently described on plasmid F it seems likely that these kil loci are involved in plasmid stable maintenance.

We have shown that the trfA gene consists of an open reading frame which produces, from two different translational starts, polypeptides of 382 and 285 amino acids, of which at least the smaller has trfA activity. The trfA gene is the second cistron of a polycistromic operon transcribed anticlockwise from a promoter at about 17.6 to 17.7 kb. The kilD locus seems to be part of this operon and may in fact be the first cistron which produces an abundant 116 amino acid polypeptide. Transcription of this 'trfA operon' is negatively regulated by the products of both the trfB/korA/korD locus (58.7 to 59.3 kb) and the korB locus (56.8 to 57.9 kb) both of which are expressed as part of a polycistronic message, transcribed from a promoter at approximately 59.3 kb, which may represent an operon autoregulated by the trfB and korB products. A third locus, incC (57.9 to 59.3 kb), is expressed as part of the trfB, korB operon and expresses incompatibility towards mini plasmids containing korB. Near the trfA operon we have identified a transcriptional unit which we believe is responsible for kilB expression because it is negatively regulated by korB but not by trfB alone.

Deletions in the $oriV_{RK2}$ region have indicated the presence of copy number control functions adjacent to and possibly overlapping with the minimal $oriV_{RK2}$ segment. The nature of these elements is being analysed at present. However, the overriding control of copy number seems to reside in the trfB and korB loci which in conjunction appear to reduce expression of the trfA gene to levels limiting for replication. The possible role of the incC locus in this copy number control circuit remains to be elucidated.

MULTIPLE INITIATION SITES FOR DNA REPLICATION FLANKING THE ORIGIN REGION OF λdv GENOME

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λdv plasmid carries four 19bp *ori* repeats in the region that is believed to be the initiation site for replication. These repeats are the target for binding of O protein, a λ-specific initiator. We have established an *in vitro* system that allows replication of any circular DNA molecule carrying the λ *ori* region. This paper describes our attempts to locate the site of primer RNA→DNA switch in the initiation process occuring at/around the *ori* region in the *in vitro* λdv replication system.

Early λdv replicative intermediates were prepared in the presence of ddCTP, an inhibitor of DNA chain elongation. Short chain DNAs produced from regions near the replication origin were purified from the intermediates, and those DNAs that are covalently linked to primer RNA were collected for mapping the transition sites from primer RNA to DNA synthesis.

Major transition sites were observed to locate on both sides of the *ori* repeats. In either side of the *ori* region, the transition sites are multiple, and are clustered in one of the two strands in such a way that DNA synthesis from either side proceeds convergently. The frequency of the "leftward" DNA synthesis is several times higher than that of "rightward" synthesis. These results reflect asymmetrical, bidirectional replication of λ.

INCOMPATIBILITY AND THE REPLICATION OF THE INCFI PLASMID COLV2-K94

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The colicin-producing plasmid ColV2-K94 is an IncFI plasmid which we have found to be capable of expressing incompatibility by both partitioning (incD-mediated) and replication control (repA-mediated) mechanisms. Two overlapping mini-ColV derivatives, pWS15 and pWS16, were obtained by digestion of the ColV plasmid pWS12 with EcoRI and BamHI, respectively. Although these plasmids contain an IncFII replicon, the intact ColV plasmid does not express IncFII incompatibility because another replicon (cloned in plasmid pWS14) functions during the plasmid's coexistence with IncFII plasmids. This second replicon shows homology with the secondary replicon of F (f7) and is dependent on the polA product of the host cell. The plasmid pWS12 behaves as a member of the IncFII group in a polA strain, BK202. The 75 kb BamHI-generated pWS16 consisted of an IncFII replicon of ColV, an entire transfer operon, and an incD partitionong locus, but lacked the second replicon. It exhibited incompatibility towards both IncFI and IncFII plasmids. The 32 kb EcoRI-generated pWS15 lacked the second replicon, the incD locus and part of the transfer operon. It was therefore non-transmissible and expressed strong IncFII incompatibility, but lacked the IncFI incompatibility properties of its parent, pWS12.

MUTANT COLE1 REPLICATION PRIMERS HAVE ALTERED SECONDARY STRUCTURES

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A ColE1 thermosensitive overproducer mutant and several revertants have been isolated. The mutant loci map within the primer RNA coding region, spanning a distance of over 300 nucleotides. The idea that the mutations alter primer secondary structure--the consequences being aberrant primer processing or function--is suggested by:
(a) altered primer RNA mobilities in non-denaturing polyacrylamide gels;
(b) nuclease T1 probing studies which indicate that new cleavage sites are exposed as a result of the mutation;
(c) computer-generated models that show a gross disruption of the most-stable wild type primer structure (at the site where RNAI presumably interacts with primer) when mutant base changes are introduced into the primer sequence.

TRANSFERABLE RESISTANCE TO CEFOTAXIME AND CEFUROXIME IN STRAINS OF KLEBSIELLA PNEUMONIAE IS GOVERNED BY A SINGLE GENE

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We extended the genetic analysis of exconjugants that originated from our transfer experiment in which Cefotaxime, Cefuroxime and Cefamandole resistance determinants could be transferred from nosocomial strains of Klebsiella to recipient E.coli K-12 strains (Knothe et al. Infection 11 : 315-318, 1983). Exconjugant colonies from the transfer plates containing either Cefuroxime or Cefamandole, were picked into minitubes with broth, and, by means of a multiloop applicator the clones were applied on to plates containing only a single drug from the multiple drug resistance spectrum of the original Klebsiella strain. Results obtained from the indirect selection analysis of more than 600 colonies obtained in five cycles of transfer to, and between, E.coli K-12 variants showed an obligatory presence of Cefotaxime, Cefuroxime, Cefamandole and Cephalotin resistance determinants in all exconjugants selected primarily with Cefuroxime, but the presence of Cefamandole and Cefalotin resistance determinants selected with Cefamandole or Cephalotin. As resistance determinant to Cefotaxime could not be separated in as many as five cycles of transfers from that to Cefuroxime, it might be concluded that, in strains studied, the transferable resistance to both these antibiotics is regulated by a single gene.

COMPARATIVE STUDIES OF COLK AND COLE1

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The natural multicopy plasmid ColK appears to be a "cousin" of ColE1. Though its genome is larger (7.2kb), its general organization of functions appears to be similar to that of ColE1. In an attempt to understand more about the structure and function of ColE1 and ColK plasmids, we have initiated a DNA sequencing study of ColK. Additionally we have been analysing, at the comparative level, the functions of ColK involved in conjugal mobility and plasmid stability.

At a functional level, the ColK and ColE1 transfer origin regions are interchangeable. A 155bp HpaII fragment of ColK was shown to contain a fully functional transfer origin, and shows a conserved DNA sequence. Similarly the mobility regions that specify proteins required for conjugal transfer are interchangeable for conjugation mediated by the IncIx plasmid R64 drdII (but not for transfer by F'lac: Tacon, W. et al, 1981). The regions equivalent to rop (Cesareni et al, 1981) and cer (Summers, D.K., Sherratt, D.J., 1984) are present in ColK.

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CONTROL OF A CHROMOSOMALLY ENCODED p-CRESOL PATHWAY BY A TOL-LIKE PLASMID IN ALCALIGENES EUTROPHUS

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A. eutrophus strain 345 metabolizes m- and p-toluate via a catechol meta- cleavage pathway. DNA analysis, curing studies and transfer of this phenotype showed that the degradative genes are encoded on an 85 kb plasmid, pRA1000. Restriction endonuclease analysis of pRA1000 showed it to be similar to the archetypal TOL plasmid, pWW0. In strain 345, pRA1000 prevented the expression of chromosomally encoded enzymes required for the degradation of p-cresol whereas these enzymes were expressed in strains cured of pRA1000. Studies with an R68.45-pRA1000 cointegrate plasmid, pRA1001, show that the gene(s) responsible for the effect on p-cresol degradation resides within or near the m- and p-toluate degradative region on pRA1000.

Analysis of the enzymes synthesized by wild-type and mutant strains and by strains cured of pRA1000, indicated that two or more isofunctional enzymes mediated several steps in meta-cleavage pathways for the degradation of m- and p-toluate, phenol and p-cresol. The formation of three 2,3-oxygenases and two semialdehyde hydrolases was indicated from an examination of the ratio of the specific activities of these enzymes against various substrates. Evidence for two semialdehyde dehydrogenases, two isomerases and decarboxylases and three hydratases was derived from the induction of these enzymes under different growth conditions. Each activity was detected when strain 345 was grown in the presence of m-toluate but not when grown with phenol or p-cresol. In strains cured of pRA1000, growth with phenol or p-cresol, but not with m-toluate, induced these enzymes.

MAPPING OF THE GENOME OF *RHODOPSEUDOMONAS SPHAEROIDES*

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Photosynthetic bacteria have proven popular subjects for studies on photosynthesis, photochemical nitrogen fixation and membrane biogenesis. The Gram negative, facultatively phototrophic bacterium *R. sphaeroides* has proven an ideal subject for combined biochemical and biophysical studies of photosynthesis. Recent advances in the genetics of this organism indicate that it is amenable to gene mapping and cloning techniques applicable to other Gram negative bacteria. Using the broad host range plasmid pRP1 carrying the mercury transposon Tn*501*, we have been able to obtain Hfr promoted chromosome mobilization; linkage results using this system of chromosome transfer enabled us to produce a map of the *R. sphaeroides* genome which consisted of two linkage groups. The ability of Tn*501* to convert pRP1 to an Hfr was thought to be due in part to its low resolvase activity, which trapped RP1 in a co-integrate with the main chromosome during the transposition of Tn*501*.

The idea that the activity of the resolvase function was critical to Hfr formation was examined using a mutant of Tn*21* deleted for its resolvase function (Tn*21* Δ *trp*R); Tn*21* is closely related to Tn*501* and forms co-integrates as part of the transposition process. The broad host range plasmid pR751 carrying Tn*21*Δ was shown to give Hfr mobilization of the main chromosome in a manner similar to RP1:Tn*501*; the major difference being that now markers on the two linkage groups (obtained with RP1:Tn*501*) show linkage to one another. It is concluded that *R. sphaeroides* has a single linkage group, presumably the main chromosome.

An additional feature of R751:Tn*21*Δ is its ability to form extremely stable co-integrates with the popular cloning vectors pBR322, pACYC184, and pUC8. Indeed the co-integrates are so stable that these vectors can be transferred and maintained (as co-integrates with pR751::Tn*21*Δ) in a wide range of Gram negative organisms including *R. sphaeroides*, *Cellvibrio mixtus*, *Aeromonas punctata*, and *Alcaligenes eutrophus*. *Rhodopseudomonas sphaeroides* genes cloned in *E. coli* using pUC8 can be transferred to and maintained in *R. sphaeroides* and the cloned segment detected by complementation of mutants of *R. sphaeroides* opening the way for more detailed analyses of the genome of this bacterium.

FUNCTION AND CHARACTERISTICS OF THICK AND THIN PILI OF TRANSFER-DEREPRESSED I COMPLEX PLASMIDS IN *E. COLI*

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IncI$_1$ and IncI$_2$ plasmids determine both thin (6 nm) flexible, and thick (10.5 nm) rigid pili. Thick I$_1$ and I$_2$ pili are structurally different. Eleven transfer-derepressed plasmids from Inc groups I$_1$, I$_5$, B, K, and Z (made in a strain with the *dnaG3* mutation) determined thin and thick I$_1$-type pili constitutively. Plasmids made with only thick pili transferred well on plates but not in a liquid (surface mating systems), those with both kinds transferring equally in both environments (universal mating systems). IncI$_2$ plasmid R721*pil*^{c}.4 (made by conventional mutagenesis) determined thin pili constitutively, but very few thick pili; its low transfer frequency (universal system) corresponded with the small numbers of thick pili. The presence of thin R721*pil*^{c}.4 pili on recipient cells converted the mating system of Sa (IncW) from surface to universal. Thus, the thin pili were only "grappling hooks" thick, rigid pili alone mediating fertile cell contact. Likewise, the thick flexible pili of R402 (IncT, universal mating system) had the same effect. All thick flexible pili probably combine the stabilizing function of thin flexible I-type pili with the fertility of rigid pili.

APPARENT AMINO ACID AUXOTROPHY MEDIATED BY E4 COLICIN PLASMIDS

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Transformation with E4 colicin plasmids, but not other E colicin plasmids, resulted in conversion of prototrophic *E. coli* K12 strain DB364 to apparent amino acid auxotrophy. Supplementation of minimal media with pools of amino acids allowed incomplete and variable amounts of growth; a pool containing Ile, Leu, Val, Met, Arg, Gly and Ser had the greatest growth-promoting effect. Prototrophic revertants were selected from auxotrophs (frequency ca. 3×10^{-4} per cell plated) without detection of plasmid loss. Analyses of the plasmids from such revertants revealed ca. 30% modified to increased sizes (> 4.7 Md), while ca. 70% remained unmodified (4.2 Md). Plasmids from prototrophic and auxotrophic clones were characterized by restriction endonuclease mapping and Southern transfers to localize the relevant plasmid sequence and ascertain the nature of the plasmid modification. Plasmids, modified via tandem duplication, were unable to mediate auxotrophy, while unmodified plasmids retained the ability to mediate auxotrophy. A chromosomal alteration was inferred to have arisen in prototrophic revertants with unmodified plasmids. Examination of detergent susceptibility and leakage of periplasmic enzymes of strain DB364 suggested a permeability barrier defect. Additional data indicated that E4 colicin plasmids perturbed their host's envelope by a mechanism independent of a putative lysis gene which, by analogy with other E colicin plasmids, may exist within the colicin operon. Furthermore, this additional envelope alteration by E4 colicin plasmids resulted in the apparent amino acid auxotrophy of strain DB364.

AEROBACTIN AND THE PATHOGENESIS OF EXTRAINTESTINAL INFECTIONS OF MAN

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Iron is essential for microbial growth. In nature, however, it exists predominantly in insoluble or complexed forms, and therefore various mechanisms have evolved in microorganisms for the specific active uptake of iron. Enteric bacteria secrete enterochelin, a high affinity catechol iron chelator (siderophore) whose synthesis and activity are controlled by chromosomal genes. In addition, some strains of Escherichia coli harbour plasmids mediating synthesis of the hydroxamate siderophore aerobactin. These plasmids significantly enhance the virulence of host bacteria in conditions of iron deficiency in experimental infections of mice.

To investigate the ubiquity of the aerobactin system among E. coli strains associated with human extraintestinal infections we have screened 451 isolates using two complementary approaches. A biological assay employs a mutant strain (LG1522) defective in both uptake of ferric-enterochelin and biosynthesis of aerobactin; isolates which secrete aerobactin (solely, or in addition to enterochelin) promote growth of this indicator strain by cross-feeding when inoculated onto a lawn on iron-deficient medium. In addition we have used colony hybridisation to detect the presence of specific nucleotide sequences directly rather than by their expression. Probes used in this screening were restriction fragments carrying aerobactin biosynthesis or receptor genes. Results show a significantly higher incidence of the aerobactin system among strains isolated from cases of septicaemia (68.8%), pyelonephritis (75.4%), lower urinary tract infections (58.4%), asymptomatic bacteriuria (61.0%), and cystitis (50.9%) than among human faecal isolates (34.3%). These data indicate that aerobactin-mediated bacterial iron uptake may be an important factor in the pathogenesis of extraintestinal infections of man.

CHARACTERIZATION OF PROMOTERS IN β-LACTAMASE MUTANTS

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A mutation in an ampicillin-resistance (Ap-r) plasmid, R6K, resulting in a tenfold increase in resistance has been analysed. Previous studies showed the original R6K plasmid had undergone intramolecular transposition to result in the duplication of the transposon, Tn2660. The two transposons were separately cloned and transposed to a small, base-sequenced plasmid, pA03, to derive the constructs pA03::Tn2660, which specifies the normal (R6K) level of Ap-r, and pA03::Tn2661, which specifies a tenfold increase in Ap resistance.

Restriction fragments which carry DNA sequences immediately 'upstream' of the initiating codon for β-lactamase were excised from each construct and used to measure the size of in vivo RNA transcripts from each construct by the Berk and Sharp (1977) method. Two new promoter sites were identified on Tn2661. Base-sequencing of Tn2660 and Tn2661 DNA showed the same sequence as Tn3, except that Tn2661 had a single base-pair substitution at coordinate 3773.

This single base change can account for the synthesis of the two new messenger RNAs identified, in one instance by the creation of an improved '-35' region, and in the other by the creation of an improved -10 'Pribnow' box. Similar experiments have verified the sites of the two β-lactamase promoters on pBR322, and a plasmid has been constructed in which the new promoter sites of Tn2661 have been substituted in pBR322 to result in transcription from four β-lactamase promoters.

ISOLATION OF A LARGE PLASMID FROM CLOSTRIDIUM THERMOACETICUM

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Clostridium thermoaceticum, a thermophilic anaerobe, ferments one mol of glucose to almost three mol of acetate. It also synthesizes acetate from CO_2/H_2 or CO. The pathway of acetate synthesis is as follows: CO_2 → formate → formyltetrahydrofolate →→ methyl-H_4folate → methyl-corrinoid. The methyl group of the methylcorrinoid with either carbon monoxide or the carboxyl group of pyruvate yields acetate. Two strains of this bacterium (one maintained in H. G. Wood's laboratory and one carried by us) have been found to contain a single large plasmid. It was isolated by the high pH technique of Anderson and McKay (Appl. Environ. Microbiol. 46, 549, 1983). It has 87 kb as determined by agarose gel electrophoresis. The number of copies of the plasmid is amplified by the addition of chloramphenicol (70 to 80 µg/ml, acridine orange 6 µg/ml, rifamycin 30 µg/ml and chloramphenicol, 30 µg/ml. The relationship, if any, of the plasmid to the acetate biosynthesis pathway or to the resistance of drugs is not clear. Efforts are being made to characterize the plasmid and to cure the bacterial strains of it.

MOLECULAR AND PHYSIOLOGICAL STUDIES OF TETRACYCLINE RESISTANCE MEDIATED BY STREPTOCOCCUS FAECALIS PLASMID pCF10

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The sex pheromone-dependent tetracycline resistance plasmid pCF10 (36.3 megadaltons) of Streptococcus faecalis has been physically mapped by restriction analysis. Southern hybridization experiments have revealed that pCF10 carries two distinct tetracycline resistance determinants. One region of pCF10 shows homology to the determinant of the non-conjugative plasmid pAMα1, whereas a second region shows homology to the tetracycline resistance element, Tn916. Our evidence indicates that pCF10 may carry the entire transposon. When wild type donor cells carrying pCF10, or mutant plasmid derivatives selected for on the basis of increased donor ability or decreased levels of tetracycline resistance, were repeatedly transferred in broth containing 10 ug/ml of tetracycline, five-to-ten fold increases in levels of resistance were observed. Wild type donor cells and some mutant strains passaged in tetracycline showed an increase in cell aggregation and plasmid transfer ability, reminiscient of sex pheromone response. In the case of the wild type pCF10, addition of 10 ug/ml tetracycline to a donor culture maintained at a density of 10^7 cfu/ml (by periodic dilution) resulted in a transient decrease in donor potential, followed by an increase of 1-2 orders of magnitude after six generations in the presence of tetracycline.

GENETIC ANALYSES OF THE VIR GENES ON THE TIC58 PLASMID OF AGROBACTERIUM TUMEFACIENS - PROMOTERS AND POLYPEPTIDES

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We have constructed a series of new plasmids that are suitable as general cloning vectors in Eschericia coli and Agrobacterium tumefaciens. Plasmids pUCD2 and pUCD206 are amplifiable in E. coli and contain a broad host range IncW origin of replication. Using these vectors we have shown that some genes in the virulence (vir) region of the tumor inducing plasmid pTiC58 are expressed constitutively in A. tumefaciens. RNA polymerase binding studies on the vir region have identified several sites of tight binding, further suggesting that there are multiple promoters within this area. We have mapped several polypeptides encoded by vir region genes using E. coli mini-cells and maxi-cells, and through a coupled in vitro transcription-translation system prepared from A. tumefaciens. The six kilobase restriction fragment KpnI-11 encodes at least six polypeptides, four of which are abolished by a Tn5 insertion. DNA sequences analysis of this cluster has shown that this polar Tn5 insertion lies within the coding region of the first polypeptide, suggesting the existance of a vir operon. Fusion of a cat gene to various positions within this gene cluster allowed us to demonstrate its constitutive expression. In contrast, cat fused to an adjacent gene was expressed only in association with plant cells.

GENES FOR ANTHRANILATE SYNTHASE IN PSEUDOMONAS AERUGINOSA ARE UNUSUALLY CONDENSED

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The adjacent genes for the two subunits of anthranilate synthase have been cloned from an R68.44 R prime plasmid originally isolated by Robert Hedges into several E. coli plasmid vectors. Initially weak expression was improved somewhat by growth rate selection in an E. coli trpE deleted host. A contiguous DNA sequence was obtained extending from 92 bp upstream to 490 bp downstream from the trpG coding sequence. The trpG gene product of P. aeruginosa itself is 200 amino acids in length and shows the five areas of sequence homology Brian Nichols finds to be conserved among glutamine amidotransferase subunits of anthranilate and p-aminobenzoate synthases. Surprisingly, the sequence does not resemble that of P. putida anthranilate synthase glutamine amidotransferase subunit any more than those of E. coli or other microbes. The sequence downstream from trpG contains no obvious candidate for an additional gene product. The sequence upstream has in one reading frame a coding sequence with 18 of 37 amino acids homologous to the C-terminus of E. coli trpE. This overlaps the trpG reading frame by 23 bp, including the termination codon (TAA). There is a typical ribosome binding sequence also included in the trpE gene about 10 bp ahead of the trpG start codon. In all, it appears that approximately 35 bp of P. aeruginosa DNA have a dual function, encoding the end of trpE and the beginning of trpG. This arrangement is sufficiently unlike the small, 1-4 bp overlaps reported in other prokaryotic operons to suggest that it may not favor translational coupling between the two gene products.

CONTROL REGION FROM PSEUDOMANAS AERUGINOSA PLASMID pMG7 WHICH REGULATES THE EXPRESSION OF THE PaeR7 RESTRICTION-MODIFICATION SYSTEM IN E. COLI

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A segment of the Pseudomanas aeruginosa plasmid pMG7 containing the restriction modification system PaeR7 has been studied. A 3.8kb DNA fragment containing both the endonuclease and methylase genes was cloned into pBR322 and expressed in E. coli. A series of Bal31 mutants have been generated from this original clone allowing for the identification of the control region of this system. The ability of these clones to restrict phage was shown to be variable depending on the segment of the control region deleted. In one clone, the expression of both the endonuclease and methylase genes is observed without restriction of invading phage.

DNA sequence analysis and subcloning experiments of this control region indicates a potentially novel regulation system for these genes.

GENETICS OF AMPLIFICATION OF TRANSPOSON TN9 IN ESCHERICHIA COLI

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We have isolated ten Tn9 insertion mutations in E. coli K12 in each of which the inserted transposon inactivates a gene required for its own amplification. These mutations, which have been designated aml, map in five well separated chromosomal loci and identify at least six different genes. They have been transferred to different genetic backgrounds and the resulting strains characterized in respect of the residual Tn9 amplification proficiency general recombination, UV sensitivity and transposition. These studies, along with investigations of the effects of different Rec mutations on Tn9 amplification, have led to the following conclusions: (i) Though wild-type alleles of all the aml genes are needed for amplification of a Tn9 located on the bacterial chromosome, several of these genes are not essential for amplification of the same transposon when it is located on the plasmid prophage of P1. It appears that a plasmid-borne transposon requires a simpler genetic apparatus for its amplification than a chromosomal transposon. (ii) Amplification of even a plasmid-borne transposon requires at least one host pathway of general recombination in active state. (iii) The aml mutations do not significantly affect UV-sensitivity, but two of them completely block the transpositional activation of the bgl operon suggesting a overlap between the transposition and amplification processes. Two of the aml mutations identify new rec genes. (iv) The F-plasmid carries genes which can amplify a chromosomal Tn9 even in the absence of functional host aml genes.

The data in support of these conclusions will be presented. Their bearing on the evolution of plasmids and a new model of gene amplification suggested by our results will be discussed.

LOCALIZATION OF A FUNCTIONAL DOMAIN IN THE COLICIN IA AND COLICIN IB MOLECULES

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The colicin Ib structural and immunity genes reside on the 60 MD low copy number plasmid Col Ib-P9. The colicin Ib structural gene has been cloned into the plasmid vector pBR322. Observations of growth characteristics of strains carrying the cloned gene and analysis of insertion and deletion derivatives of the recombinant plasmid have led to the conclusion that colicin Ib production can occur without the colicin Ib immunity system. The direction of transcription for the colicin Ib gene was determined by use of an expression vector and subsequently confirmed by DNA sequence analysis of the colicin Ib promoter. The promoter was found to contain a sequence homologous to the base sequence of the binding site for Lex A protein which is involved in "SOS" regulation. Hybrid genes were constructed by ligating cloned fragments of the colicin Ib gene with fragments of the cloned colicin Ia gene and transforming them into E. coli. From an analysis of the immunity specificities of the chimeric colicins and the locations of the fusions, we concluded that the information necessary for immunity recognition by colicin Ia and colicin Ib resides in the C-terminal half of the colicin molecules.

FREQUENCY OF THE TetA, TetB, AND TetC CLASSES IN SALMONELLA AND SHIGELLA STRAINS ISOLATED IN MEXICO CITY

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In this work, we determined the level of induced tetracycline (Tc) resistance, the colicin production, the plasmid content, and the frequency of classes TetA, TetB and TetC in 20 Salmonella and 34 Shigella clinical strains isolated in Mexico City in 1978-1979. All the strains were resistant to streptomycin (Sm), Sulfonamide (Su) and Tc.

Our results showed that there were differences in the level of induced Tc resistance, colicin production and plasmid band pattern not only in Salmonella and Shigella strains, but also between the Sh. sonnei and Sh. flexneri strains. The class most frequently present in the Salmonella and Sh. flexneri analyzed was TetB. A high percentage of Sh. sonnei (73%) did not hybridize with the Tet classes tested, the majority of these strains were resistant to low Tc concentrations (25 µg/ml). The TetD class, represented by the plasmid RA1, conferred resistance to high Tc concentrations to E. coli and Sh. sonnei. This fact suggests that the class or classes of Tet genes present in Sh. sonnei were different from the four classes reported in Gram-negative bacteria.

CONSTRUCTION OF A SINGLE STRANDED DNA PLASMID CONTAINING A PROMOTER FOR SP6 RNA POLYMERASE

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A major obstacle in studying biochemical processes involving RNA transcripts has been the difficulty in obtaining sufficient quantities of purified RNA species. Two recently developed promoter plasmids, pSP64 and pSP65, are capable of directing in vitro transcription of large amounts of unique RNA. The value of these plasmids lies in the high specificity of purified bacteriophage SP6 RNA polymerase for the SP6 promoter DNA, and the multiple cloning site, oriented in both directions, immediately following the promoter. To further extend the usefulness of these plasmids, it would be advantageous to obtain them as single stranded DNA (ssDNA) for mutagenesis and rapid sequencing purposes. We have constructed four ssDNA promoter plasmids by inserting a 514 bp restriction fragment containing the replication origin and morphogenetic signal of the ssDNA bacteriophage f1 into pSP64 and 65, in both orientations. Because the orientation of the f1 fragment determines which of the two DNA strands of the plasmid will be packaged as the viral particle, the resulting plasmids, pUI+4 and +5, and pUI-4 and -5, are secreted as either sense or anti-sense strands, respectively, relative to the RNA transcript. Secretion of the pUI vectors as ssDNA upon superinfection with the interference resistant phage IR1 has been demonstrated by agarose gel electrophoresis, the ability to transduce the ampicillin marker, and dideoxy sequencing. We are presently cloning cDNA to parathyroid hormone (PTH) mRNA in pUI+4 and -4, for subsequent in vitro transcription and translation. In addition to the more traditional plasmid DNA manipulations possible with the pUI vectors, we will apply ssDNA mutagenesis techniques on either strand of the PTH insert and rapidly confirm the change by dideoxy sequencing. Thus, microgram quantities of mutant PTH RNA transcripts will be rapidly synthesized for subsequent functional analysis.

GENETIC PROPERTIES OF LARGE PLASMIDS CONTROLLING RESISTANCES TO HEAVY METALS IN *ALCALIGENES EUTROPHUS CH34*

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A. eutrophus CH34 is a facultative chemolithotroph carrying two large plasmids : pMOL28 (165 kb ; resistances to Cobalt and Nickel) and pMOL30 (220 kb ; resistances to Cadmium, Cobalt and Zinc). These plasmids poorly transfer from the wild type strain to the plasmid free derivate AE104, the frequencies being around 10^{-7} transconjugant per donor cell. The transfer of both plasmids was strongly stimulated by IncP1 plasmids as RP4 and pUZ8 (52 kb ; Km, Tc, Hg). RP4 mobilized either pMOL28 or pMOL30 at frequencies up to 10^{-3} transconjugant per donor cell.

DNA from nickel-resistant and zinc-resistant transconjugants obtained with RP4 or pUZ8 helper plasmids was analyzed in agarose gel electrophoresis according to Kado & Liu.

The RP4 mediated Ni^r and Zn^r transconjugants contained the pMOL28 or pMOL30 plasmid and all of them also carried an enlarged RP4 plasmid. Some of these became also sensitive to tetracycline. Rearrangements including cointegrates, deletions or insertions in pUZ8 were also observed in pUZ8 mediated Zn^r or Ni^r transconjugants.

We analyzed by digestion with endonucleases three insertions in RP4 and one in pUZ8, all coming from Zn^r transconjugants : all of them contain a similar HindIII fragment (6 kb) and a similar EcoRI fragment ($\sim$ 2.5 kb). It suggests the existence of a common transposable element. One of these insertions in RP4 was mapped inside the 2 kb KpnI-BglII fragment of RP4 thus near oriV. Insertions in RP4 or pUZ8 isolated from Ni^r transconjugants have a distinct restriction pattern.

pUZ8 plasmids which have received an insertion in Ni^r transconjugants are also able to mobilize at frequencies of 5.10^{-7} R1-Cm, a 5 kb Tra^- Mob^- plasmid derived from IncFII plasmid R1 (L.Roueach, pers.comm.)pUZ8 is unable to mobilize this plasmid ($< 10^{-8}$).

These results suggest the presence of different transposable elements in pMOL28 and pMOL30.

DNA SEQUENCES OF THE MERCURIC RESISTANCE GENES OF PLASMID R100 AND TRANSPOSON Tn*501*

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Five genes in the mercuric resistance operon of plasmid R100 have been identified by Tn*5* insertion mutation studies (ref. 1). A comparison of DNA sequence data from R100 with that from Tn*501* show close homologies in the plausible reading frames for the genes predicted from the genetic studies (ref.2). The sequence in the operator promoter region is most highly conserved. There are many wobble changes in the coding regions. The *merR* gene contains 180 base pairs both in R100 and Tn*501*. There are seven amino acid changes in these arginine-rich serine-rich *merR* polypeptides. The *merT* gene codes for a very hydrophobic polypeptide of 116 amino acids and the *merC* for a polypeptide 91 amino acids long with a hydrophobic amino-terminal end. Whereas, the *merR*, *merT* and *merC* genes are of the same length in Tn*501* and R100, the *merA* genes for the mercuric reductase enzyme subunit (1.7 kb) differ slightly in length. Comparisons within the reading frames indicate an approximately 85% base pair identity between the R100 and the Tn*501* systems with approximately 90% amino acid identities. However, within the genes some regions are more conserved than others.

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GENETIC ENGINEERING WITH ACETOGENS AND METHANOGENS USING PLASMIDS AND PHAGES

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Both methanogens and acetogens constitute of several industrially important bacteria, which are source of useful chemicals and provide with means of studying microbial physiology and genetics in fascinating ways. Little attempt has been made to genetically engineer these organisms for desired and higher yields of chemicals. Physiologogical limitations of these organisms may also be genetically manipulated to enable them to produce more and desired chemicals, and make them to grow autotrophically or on agricultural wastes.

Molecular techniques have been applied to species of *Methanosarcina* and *Methanobacterium*(Reeve 1981). Cloning of genes from methanogens using phage lambda and cosmids are being attempted. Plasmids occur in *Halobacterium*(Thom 1983). *Clostridium butyricum* and *C. aceobutylicum*(Minton and Morris 1981, Truffaut and Sebald 1983) contain plasmids. Transfer of genetic information between species of clsotridia have been detected by Magot(1983). Genes from species of *Clostridium* have been cloned in phages and made to get expressed in *E. coli*. Phages of clostridia have been discussed by Ogata and Hongo(1979), and Reid et al.(1983). Attempts are being made elsewhere also to isolate phages and plasmids from acetogens.

The interesting and useful enzyme complexes of these organisms make them very promising systems for applying recombinant DNA technology, plasmid biology and genetic engineering to obtain basic scientific informationon on physiology and genetics of acetogens and methanogens, especially thermophiles; as well as their potential use in industrial processes.

The enormous scope in this area has not been fully anticipated, and there are definite limitations due to the lack of proper experimental techniques related to these unusual organisms, that hinders breakthroughs in genetic engineering with these important group of anaerobes and thermophiles.

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SELECTIVE EXPRESSION OF A PLASMID GENE DURING BACILLUS SUBTILIS SPORULATION

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The transition of Bacillus subtilis from vegetative growth to sporulation is accompanied by changes in the predominent forms of RNA polymerase within the cells. The various enzyme forms differ in the sigma-like subunits that associate with a common core enzyme, and at least four forms of RNA polymerase have been identified in vegetative and early stationary phase cells. As cells enter sporulation a sporulation-specific RNA polymerase form, sigma-29, appears. We have observed that the cat-86 gene in plasmid pPL603, which specifies chloramphenicol acetyltransferase, is selectively expressed in Bacillus subtilis at a stage in sporulation in which internal spores are first observed (approximately T8). The gene is unexpressed in vegetatively growing cells. cat-86 expression and spore formation are both blocked when cells are grown in excess glucose. cat-86 expression at T8 is due to selective transcription of the gene, since cat-86 mRNA is undetectable in vegetatively growing cells, but is readily demonstrated in sporulating cells. The transcription start site for cat-86 mRNA from sporulating cells is within a 203 bp restriction fragment designated P1, which is located upstream from the cat coding region on pPL603. Deletion of P1 from pPL603 eliminates the sporulation-associated expression of cat-86. Host sporulation genes whose function is absolutely required for cat-86 expression at T8 include six early sporulation, spo0, genes and spoIIE. pPL603 therefore provides the first system in which the in vivo expression of a known, plasmid-linked gene is dependent on sporulation-specific changes in B. subtilis.

REGULATION OF TRANSCRIPTION INITIATION OF *xyl* OPERONS ON TOL PLASMID

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The TOL plasmid from *Pseudomonas putida* mt-2 specifies enzymes for complete degradation of toluene and xylenes. The genes for the enzymes are organized in two regulatory blocks. The *xylABC* operon directs the synthesis of enzymes for conversion of toluene to benzoate, and the *xylDEGF* operon for complete degradation of benzoate or *m*-toluate. Analyses using the molecular cloning technique revealed the following regulatory mechanism of gene expression: The gene product of the regulatory gene *xylR* activates the *xylABC* operon in the presence of *m*-xylene or *m*-methylbenzyl alcohol. The activation of the *xylDEGF* operon is achieved by the product of another regulatory gene *xylS* in the presence of *m*-toluate. Both *xylR* and *xylS* products are required for the *m*-methylbenzyl alcohol-dependent activation of the *xylDEGF* operon.

The nucleotide sequence of the regulatory region of each operon was determined. The transcription initiation sites *in vivo* were determined by S1 nuclease and reverse transcriptase mapping. The transcription of the two operons in *Escherichia coli* started exactly at the same site as in *P. putida*, and the amounts of mRNA synthesized in *E. coli* were substantially lower than those in *P. putida*.

The nucleotide sequence of *xylABC* promoter showed some homology with *xylDEGF* promoter in -10 and -35 regions. These sequences, however, were not homologous with the consensus sequences of -10 and -35 regions found in *E. coli* promoters. A sequence complementary to the 3' ends of 16s rRNA of *Pseudomonas aeruginosa* and *E. coli* existed in front of the predicted start codon of the first gene of each operon. These results indicated that the inefficient expression of TOL genes in *E. coli* is due to the poor start of transcription.

PLASMID AND CHROMOSOMAL INDOLEACETIC ACID GENES OF *PSEUDOMONAS SAVASTANOI*

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Pseudomonas syringae pv. *savastanoi* is a plant pathogenic bacteria which produces indoleacetic acid (IAA). The production of IAA by the bacteria is a requirement for the induction of galls in the host plants, olive and oleander. Tryptophan is converted to IAA by *P. savastanoi* in two steps with indoleacetamide as the intermediate. In strains of *P. savastanoi* isolated from oleander galls the genetic determinants for IAA production are located on plasmid DNA. Yet in strains of the bacteria isolated from olive galls, these genes are located on chromosomal DNA. From different oleander isolates three unique plasmids of 52, 73, and 70 kb (pIAA1, pIAA2 and pIAA3 respectively), have been found which carry the IAA genes. The region of DNA encoding the IAA genes has been cloned in a wide host range plasmid. Evidence obtained from restriction enzyme disgests and southern blot analysis indicate that these plasmids contain large regions of homologous DNA. These experiments show that the region of DNA which contains the IAA structural genes (and possibly other functions) is conserved in the three plasmids. There appears to be little or no difference between the IAA synthesis genes from the different plasmid and the chromosomal isolates of the bacterium. (Aided by grants from the USDA and NSF).

PLASMIDS OF A NOVEL TYPE FROM PENICILLINASE PRODUCING HAEMOPHILUS INFLUENZAE

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H.influenzae, as a causitive agent of meningitis, septicemia, and epiglottitis in infants, is an important pathogen. Since 1975, penicillinase producing strains have been reported throughout the world; these strains are characterized by two types of plasmids : those belonging to the first type are larger than 45 kb and share a common core; those belonging to the second type are 7.4 kb large and are similar to the resistant genetic elements found among N.gonorrhoeae; both types of plasmids carry transposons of the Tn3 family or deletion derivatives of them.

During an epidemiological survey of the resistant H.influenzae in Switzerland, two penicillinase producing clinical isolates characterized by a novel type of resistant plasmid wer found, one in Bern and the other in Geneva. These genetic elements, pPJ301 and pPJ302, are 10.0 kb large and carry Tn3-like transposons as demonstrated by transposition assays on λ DNA, heteroduplex analysis, and Southern experiments using TN2301 (a Tn3-like transposon) as a probe. A restriction map with different endonucleases has been constructed. Transformation experiments showed that pPJ301 and pPJ302 can replicate in E.coli; however, when introduced into E.coli C600, these plasmids frequently originate larger derivatives characterized by DNA insertions up to 1.0 kb. Work is in progress to analyse the nature of these insertions so as the origin of these plasmids of a novel type in H.influenzae.

TRANSPOSON MUTAGENESIS AND CLONING STUDIES OF A CITRATE-UTILIZATION DETERMINANT FROM A PLASMID IN *ESCHERICHIA COLI*

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Atypical *E. coli* strains that can utilize citrate as sole carbon source frequently harbor plasmids with the gene(s) governing citrate utilization. These plasmids code for a citrate transport system (C.H. Reynolds and S. Silver, 1983, J. Bacteriol. 156: 1019-24). The citrate utilization determinant from a 200 kilobase (kb) natural plasmid was cloned as a 9 kb *Pst* I fragment (designated pWR61). Citrate-non-utilizing mutants of pWR61 were isolated with transposon Tn*5* and the physical location of the insertions determined by restriction endonuclease mapping. Then, the citrate utilization determinant was subcloned as 4.7 kb *Pst*I-*Eco*RI fragment (pMS185). To locate the citrate utilization determinant more precisely, derivatives of pMS185 were constructed. The citrate determinant is located in a 1.7 kb *Bam*HI-*Bgl*II fragment. DNA sequencing analysis of the citrate determinant is proceeding now by methods using M13 phage. ^{14}C-citrate transport properties of these mutants were determined to correlate genetic and physiological characteristics.

CONSTRUCTION OF CHIMERIC OPERONS FOR SPECIALTY CHEMICAL PRODUCTION

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Combining genes from different microorganisms and controlling their expression with a common regulatory element is a promising route to the biosynthesis of chemicals not ordinarily produced by any single microorganism. We have used this approach to combine the naphthalene dioxygenase genes from *Pseudomonas putida* with the tryptophanase gene from *Escherichia coli* to produce the dye indigo. The action of tryptophanase produces indole, which is oxidized to an indigo precursor by the naphthalene dioxygenase enzyme complex.

Construction of the Indigo Operon involved the cloning of 5 genes: the 4 structural genes for the components of naphthalene dioxygenase, namely the flavoprotein, the ferredoxin, and the two subunits of the iron-sulfur protein and the structural gene for tryptophanase. Constructions were studied with the Indigo Operon under the control of the *lac* promoter. Higher enzyme levels were observed from promoter-proximal genes than from promoter-distal genes. The highest level of indigo production was observed when the tryptophanase gene was placed immediately downstream from the *lac* promoter followed by the naphthalene dioxygenase genes. Our results suggest that designing chimeric operons for specialty chemical production may be a feasible alternative to current chemical syntheses.

TOXEMIA DE LA PREÑEZ EN OVINOS-TRATAMIENTO MEDIANTE

HISTEROTOMIA EXCLUSIVAMENTE

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La experiencia se realizó en una majada de 26 ovejas Corriedale y un carnero, en los que se detectó la epizootia. El objetivo fué comprobar los resultados obtenidos unicamente mediante operación cesarea, sin complementos terapeuticos.

Se utilizó instrumental común de cirugía, practicandose anestesia epidural con 10 cc de Novocaína al 2%. Se realizó incisión en región ventro-lateral izquierda, siendo el tiempo promedio que insumió cada operación de 12-14 min. La incidencia fué del 30, 7%. De los 8 animales operados, 3 vivieron (37, 5%) y 5 murieron (62, 5%) entre las 16 y 36 hs. posteriores a la cesarea.

Todas las enfermas eran melliceras en el último mes de gestación. En todos los casos se intervino quirurgicamente en el último período de la enfermedad cuando ya había decúbito permanente, pedaleo, opistótonos, rechinar de dientes y sialorrea.

Los resultados se consideran satisfactorios y es de esperar que la letalidad sea menor actuando ante los primeros signos.

STABILITY FUNCTIONS OF COLE1

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The high copy number plasmid ColE1 which appears to be partitioned randomly at cell division, requires two independent functions for its stable maintenance. The first (*cer*) is a region of less than 280 bp required in *cis* for the resolution of plasmid multimers to monomers. Multimerisation of *cer*$^-$ plasmids by homologous recombination reduces the number of segregating units per cell and hence causes instability (Summers & Sherratt, 1984). *cer* contains the site for *trans*-acting functions encoded by the chromosome of many *E. coli* strains and by ColE1 itself.

The *rop* protein (Cesareni *et al*, 1981), in addition to its activity as a repressor of plasmid replication, is also required for the stable maintenance of ColE1. *rop*$^+$ plasmids are more stable than their *rop*$^-$ counterparts, even though their copy number is lower. We propose that, in addition to reducing mean copy number, *rop* reduces the copy number variance in a cell population thus removing a sub-population of low copy number cells which would otherwise generate plasmid-free segregants at relatively high frequency.

Cesareni, G., Muesing, M.A. & Polisky, B. (1981). Proc. Nat. Acad. Sci. USA **79**, 6313-6317.

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THE ROLE OF PLASMIDS IN GROUP N LACTIC ACID STREPTOCOCCI FROM DAIRY STARTER CULTURES

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Lactic acid streptococci are needed for the production of fermented milk and cheese. The following functions are linked to plasmids (3): transport and metabolism of lactose, transport of citrate, proteolysis of caseins, protection against bacteriophages by restriction/modification and synthesis of bacteriocins. 2 to 15 different plasmids in sizes from 1 to 80 Mdal have been detected in 400 strains (1). The plasmid profile of a strain is stable and strain-specific in milk, allowing determination of strain composition of single, multiple and mixed strain cultures. European mixed cultures do contain up to 50 strains distinguishable by their plasmid complement. Plasmids can be exchanged by conjugation, transformation of protoplast and for smaller plasmids, by transduction (2). Many of the plasmids are cryptic but hybridize with a bacteriocin plasmid indicating homologous DNA and common genetic elements (4). The mechanism by which the large number of plasmids has evolved in these bacteria remains to be established.

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FUNCTIONS OF EXTRACHROMOSOMAL DNA IN THE EPIPHYTIC BACTERIUM *ERWINIA UREDOVORA*

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Erwinia uredovora is an epiphytic yellow pigmented bacterium, related to E. herbicola. The strains 20D3 and 20D31 contain 4 and 6 extrachromosomal DNA respectively, with molecular sizes ranging from 7.8 to 260 kb. Functions ascribed to some of these plasmids have been analysed.

1. The appearance of unpigmented (Flv^-) and Thi^- phenotypes upon curing treatments is correlated with the loss of the 260 kb plasmid (pULG1 in 20D3, pULG11 in 20D31) in both strains. The pigment lost after curing has spectral characteristics of carotenoid. pULG1 and pULG11 carry genes related to the biosynthesis of the thiazole moiety of thiamine.

2. A phage tail like bacteriocin active against E. amylovora and E. carotovora strains can be induced in both strains by mitomycin C. This induction is correlated with the amplification of an extrachromosomal DNA of 17 kb coding for bacteriocin determinants. The whole phenomenon suggests a prophage induction.

3. A 88 kb plasmid (pULG3) from 20D3 was marked by the phage D108.1 (a miniderivative of D108 carrying the marker Cm^r) and found to be self-transferable. pULG3 belongs to the Inc M group.

4. Through curing and mobilization, a 28 kb DNA from 20D31 has been identified as a conjugative plasmid conferring susceptibility to the bacteriophage GU5. This plasmid (pULG14) belongs to the Inc N group. It is one of the smallest natural conjugative plasmids yet described in gram-negative bacteria.

MOLECULAR CLONING AND EXPRESSION OF A PLASMID DNA REGION INVOLVED IN VIBRIO ANGUILLARUM IRON UPTAKE

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Plasmid pJM1 harbored by Vibrio anguillarum mediates a very efficient iron uptake system associated with the high-virulence phenotype of this bacterium. Transposition mutagenesis analysis allowed us to localize the iron uptake region on pJM1. Iron-uptake deficient mutants generated by transposition mutagenesis were characterized as having lesions in the biosynthesis of either a siderophore or a membrane receptor for the complex siderophore-iron. Further characterization of the pJM1 iron uptake region required the molecular cloning of essential components of this system. Subsets of the iron uptake region were cloned in the cosmid pVK102, a derivative from the wide host range cloning vehicle pRK290, in vitro packaged and transduced into Escherichia coli. The recombinant DNA molecules were then mobilized to various iron uptake deficient strains of V. anguillarum and scored for their ability to grow under conditions of iron limitation and to express siderophore and/or receptor activity. The results showed that concomitant with the receptor activity expressed by exconjugants harboring the recombinant cosmids, there was induction of the biosynthesis of the protein OM2 under iron limiting conditions. OM2 is an 86 kd outer membrane protein which, thus, may be the presumed receptor. Some of the exconjugants harboring the cloned derivatives were able to produce siderophore activity. Iron uptake proficient derivatives obtained after introduction of the cloned pJM1 iron-uptake region regained full virulence, demostrating the intimate relatin between these two properties.

CHROMOSOMAL LOCALIZATION OF AEROBACTIN-MEDIATED IRON UPTAKE GENES COMMONLY ENCODED BY CERTAIN COL V PLASMIDS.

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The aerobactin-mediated iron uptake system encoded by the pColV-K30 and other Col V plasmids has been associated with the ability of invasive Escherichia coli strains to cause disease. To study the occurrence of these genes in microorganisms responsible for nosocomial human neonatal infections, we have examined a number of enteric strains isolated from cerebrospinal fluid of human neonates. The clinical isolates E. coli K 1 CHBA187, Enterobacter cloacae CHBA31 and Klebsiella pneumoniae CHBA33 exhibited a functional aerobactin-mediated iron uptake system, as assessed by a cross-feeding bioassay and by their sensitivity to cloacin, a bacteriocin that recognizes the outer membrane receptor for iron-aerobactin complexes. By using a variety of techniques we could not find any plasmid harboring the aerobactin genes in these strains. Hybridization of restriction endonuclease-cleaved chromosomal DNA from these isolates with various clones containing subsets of the pColV-K30 aerobactin region showed a chromosomal location for these genes. Analysis of internal sequences demonstrated that the chromosomal aerobactin genes were conserved as compared with those present in the pCol V-K30 plasmid, although they were located on a different restriction site environment. The flanking sequences of the aerobactin genes in pColV-K30 were recently shown to be IS1-like elements (Perez-Casal and Crosa, 1984, submitted for publication). Probes containing IS1 sequences hybridized with the same chromosomal restriction endonuclease fragments that harbored the aerobactin genes. The finding of flanking and internal sequences of aerobactin genes on plasmids and chromosomes suggest a recombinational mobility of these genes which may have played a role in their epidemiological spread.

ISOLATION OF THE A. TUMEFACIENS TUMORIGENIC SUPPRESSION GENES ON THE IncW R-FACTOR pSa

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We have previously reported that the intracellular presence of the IncW plasmid pSa prevents Agrobacterium tumefaciens from inciting crown gall tumors on permissive host plants Loper and Kado (1979) J. Bacteriol. 139: 591-6., Farrand et al. (1981) Mol. gen. Genet. 181: 44-51., Tait et al. (1982) Mol. gen. Genet. 186: 10-15 . This phenomenon is absolute for a range of plant species tested, and operates in both octopine and nopaline strains of A. tumefaciens. To further examine the role of pSa in virulence suppression, it was necessary to first clone the R-factor genes involved. To this end, a 7.6 kb segment of pSa was isolated first in pUC4 as a partial Sau3A fragment and then transferred as an EcoRI fragment to pUCD105, a stable Agrobacterium-E. coli shuttle vector containing the replication and stability regions of the plasmid pTAR (see abstract by Gallie et al.). This DNA fragment retains all information required for complete virulence inhibition in vivo; no accessory pSa sequences are necessary. The suppressive DNA fragment is presently being characterized with respect to gene number and location, as well as the encoded polypeptide products. We have also found that the IncW plasmid R388 encodes biological virulence suppression, and that the related plasmids R7K and R404 contain sequences homologous to the 7.6 kb DNA fragment of pSa. This evidence indicates that virulence suppression may be a common feature of many IncW group plasmids.

SINGLE-STRANDED DNA BINDING PROTEIN ACTIVITY CODED BY PROMISCUOUS PLASMIDS

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The plasmid F has been shown to confer single-stranded DNA binding (ssb) activity on its host (Kolodkin *et al* 1983). The biological role of this ssb protein is not known: it is not coded by the *tra* operon of F, although it would be expected to be involved in protecting the separated DNA strands formed during conjugation. We have shown that the promiscuous plasmid RP4 encodes a DNA primase in its Tral region that is involved in the priming of the DNA strand transferred to the recipient during conjugation (Lanka and Barth, 1981). A 12kb fragment carrying the primase gene has been cloned (by A. Merryweather in our collaboration with B. Wilkins) from the RP4::Tn7 derivative pRP26 which overproduces primase. The vector was the multicopy promiscuous plasmid R300B. This clone (pAM4) completely suppresses the temperature-sensitive phenotype of an *E.coli ssb* mutant (KL450) at 44°C while the plasmid-free strain loses viability by over four orders of magnitude. RP4 itself suppresses ssb^{ts} but not as completely as pAM4. The vector R300 also gave suppression, by over two orders of magnitude.

As DNA primases presumably bind to single-stranded DNA, the ssb activity of RP4 may be due to its primase. R300B (like F) has no measurable primase activity however. The regions responsible for this suppression on the two promiscuous plasmids will be characterized and mapped.

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CHROMOSOMAL INTEGRATION AND EXCISION OF A MULTICOPY PLASMID JOINED TO PHAGE DETERMINANTS FOR SITE SPECIFIC RECOMBINATION

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We are studying the life style (cytoplasmic *vs.* chromosomally integrated) of derivatives of the small, multicopy plasmid pBR322 into which a 4.6 kb fragment of temperate bacteriophage P2 DNA has been inserted (Ljungquist, E. and Bertani, L.E., 1983, Molec. Gen. Genet. 192:87-94). This fragment contains P2 genes *int* (integrase), D and U (tail proteins) and site *att* (attachment). Gene *cox* (control of excision) is absent from this fragment. The degree of amplification of the plasmid has been followed indirectly by the level of resistance to tetracycline and the degree of complementation upon infection with a gene D (or U) defective bacteriophage.

Previous observations (Bertani, G., Ljungquist, E. and Bertani, L.E., 1981, Abstract, IUPAC Congress, Vancouver, B.C.) suggested that such plasmids could integrate chromosomally and that strains of normal viability, apparently carrying a single integrated rather than multiple cytoplasmic copies of the plasmid, could be obtained by a rather laborious procedure.

We have now confirmed these results using a *ts-polA* host strain of *Escherichia coli* C: growth at the non-permissive temperature eliminates the cytoplasmic plasmid population, while a relatively high proportion of the bacteria retain low levels of tetracycline resistance and of gene D complementation. These strains are generally stable even when cultured at low temperature (host cell phenotypically $polA^{+}$). If infected with a temperate P2 phage, however, expression of genes *int* and *cox* appears to lead to mobilization of the integrated plasmid and to the reestablishment of a cytoplasmic plasmid population. If the fragment carries a defective *int* gene, chromosomal integration does not take place spontaneously, but does occur when the P2 *int* gene product is supplied by a helping phage.

This work aims at eventually developing a method for transfer of foreign genes directly to the bacterial chromosome and their amplification *in situ*. It is sponsored by the Energy Conversion and Utilization Technologies Division of the U.S. Department of Energy, through an agreement with the National Aeronautics and Space Administration.

THE PLASMID ColE1. GENETIC ORGANIZATION AS DERIVED FROM ITS COMPLETE NUCLEOTIDE SEQUENCE AND RNA POLYMERASE BINDING STUDIES.

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The primary structure of the 6646 basepairs plasmid ColE1 has been determined. The plasmid genome can be divided into eight functional regions: cea-kil(structural gene for colicin E1 and its possible release factor), imm(colicin E1 immunity), bom(basis of mobility), mob(plasmid mobilization), exc(plasmid entry exclusion), rep(replication functions including the origin of DNA replication and the RNA primer coding region), inc(plasmid incompetibility and copy number control), and rom(modulation of RNA I activity). From the nucleotide sequence, over twenty open reading frames can be identified. Overlapping of these coding sequences occured at three areas including the rep, mob and cea regions. Overlapping transcription units in opposite orientations also existed in the cea-kil/imm, mob/rom, and rep/inc regions. Regulation of gene expression in ColE1 is also extremely complex. These included regulation by the SOS regulon, cAMP receptor protein, possible attenuations and transcription antitermination in the cea-kil, exc, and mob operons. The locations of known and potential promoters, ranging from having very strong to very weak affinity to bind Escherichia coli RNA polymerase holoenzyme, have been mapped by a combination of nitrocellulose filter binding assays, DNase I "footprinting", and exonuclease III mapping. The overlapping of transcription and coding sequences in ColE1 showed unusual economy in genetic organization and conservation of DNA sequence.

P1 MINI PLASMIDS PRODUCED IN VIVO

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E. coli recA, dnaA(Ts) strains integratively suppressed by P1 cop mutants (copy number 5-8 times P1 wild type) contain only a single integrated copy of the P1 prophage when the cells are grown at the nonpermissive temperature for dnaA (Froehlich et al, 1983). When the P1cop-suppressed strains are shifted to 30°C, the survival frequency is very low (10^{-3} to 10^{-5}). 90% of the 30° survivors contain supercoiled plasmid DNA as well as the integrated copy. Southern Blot analysis indicates that the integrated copy is whole P1. Restriction analysis and Southern Blot analysis show the plasmid DNA is present at high copy number. There is no common terminus in the P1 miniplasmids analyzed, indicating that they are not formed from the chromosomal P1 by recombination at a unique site. Probably, the miniplasmids are produced by premature termination of replication rounds initiated from the origin of the integrated P1.

All of the P1 miniplasmids contain the DNA region believed to serve as the P1 plasmid origin. Some of the miniplasmids are independent replicons and others are not. Those that are not independent replicons persist in cells carrying appropriate cloned fragments of P1. This allows us to define a minimal replicon and to define a DNA region encoding a trans-acting product required for replication.

THE INTERACTION OF MINI-F WITH THE PLASMIDS R124 and R124/3

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The Inc FIV plasmid R124 encodes a unique restriction and modification (R-M) system. R124/3 is a derivative plasmid which encodes a R-M system of different specificity. When R124 or R124/3 are present in the same cell as the sex Factor F a restriction deficient (r^-) phenotype results. This r^- phenotype reverts back to r^+ with a frequency dependent upon the particular isolate. When the F plasmids are isolated from these reverted stains they are found to encode the R-M system of the donor R124 or R124/3 plasmid. The R-M system is translocated into the region of EcoR1 fragments 5 and 7 of F, which encodes the Phi and Ori VI functions of F. During this transfer of DNA some F DNA is lost.

Recent work with mini-F (cloned EcoR1 fragments 5 and 7 of F) have shown that they alone do not result in the translocation of R-M genes from R124 or R124/3. EcoR1 fragments 5 and 7 of F when present in the same cell as R124 or R124/3 result in a fraction of the cells expressing a r^- phenotype which reverts back to a r^+ phenotype. This reversion process is not accompanied by any DNA transfer or rearrangement between F, R124, or R124/3 plasmids, This strongly suggests that some other region of F is involved in the DNA rearrangement.

Studies with mini-F have also shown that under certain circumstances the rearrangement results in R124 expressing incF1 incompatability instead of the normal Inc FIV.

THE STRUCTURE AND FUNCTION OF MINI-Rts1

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Rts1 is a large(ca. 200 Kb) stringent type plasmid of the Inc group. The basic replicon of Rts1, named mini-Rts1, is capable of autonomous replication in a polA Escherichia coli host and is main tained stably at 37°C in a low copy state(about 3 copies per host chromosome).

We determined the nucleotide sequence of mini-Rts1 consisting of 1,855 base pairs. Two clusters of direct repeated sequences wer found on the mini-Rts1 genome, each of which contained five 24-bp repeats(left cluster) and three 21-bp repeats(right cluster). A large open reading frame that directed from right to left and coul encode a 32,980 dalton polypeptide(RepA protein) consisting of 288 amino acids was located between the two clusters. A 33-Kd protein corresponding to the RepA protein was detected in the E. coli mini cell system. Outside the right cluster, an oriC homologous region consisting of four GATC sequences accompanying TTATCCACA sequence was located.

All the eight repeats contained a consensus sequence "TTCCCC-PyPyPuPuCACACACC", and both cluster-containing fragments expressed strong incompatibility toward mini-Rts1, respectively. A fragment containing the five repeats of the left cluster exhibited a much stronger incompatibility than the four repeats-containing fragment suggesting that the strength of incompatibility is dependent on th number of the repeating unit. In addition, the copy number of the mini-Rts1 plasmid increased three fold upon removal of the four repeats-containing region from the plasmid. Thus, the direct repea of mini-Rts1 appear to express incompatibility and copy control functions by titrating the RepA protein or other component(s) bein essential for the plasmid replication. The overall structure and function of mini-Rts1 are quite similar to those of mini-F and min P1.

A considerably high copy mutant(about 20 copies per host chro-mosome) was constructed by removing the four repeats from the "cop mutant of mini-Rts1, on which two base pair substitutions occurred i.e. at the RepA coding region and near the oriC homologous region of mini-Rts1.

STREPTOMYCES PROMOTER-PROBE PLASMIDS AND CARTRIDGES

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The ribosome binding site and coding sequence of the kanamycin phosphotransferase gene of the E. coli transposon Tn5 can be separated from the promoter by cleavage with the restriction endonuclease BglII or BclI which cut 35 and 30 base pairs, respectively, ahead of the ATG start codon. Streptomyces plasmids carrying the promoter-less resistance determinant conferred kanamycin resistance on their host. The level of resistance varied between c. 2 and 100 μg kanamycin/ml, depending on the position and orientation of the insert. Addition of a 333 base pair Sau3A fragment containing a transcription terminator of phage fd in front of the kanamycin resistance determinant effectively abolished kanamycin resistance in Streptomyces, thus supporting the hypothesis that expression of resistance depends on upstream promoter activity. Promoter-probe plasmids (e.g. pIJ425) with a unique BglII cloning site between the kanamycin resistance gene and the fd terminator are useful for the isolation of DNA fragments with in vivo promoter activity from Streptomyces strains.

Transcriptional activity can also be detected by inserting the promoter-less kanamycin resistance fragment into DNA sequences of interest. Experience has shown that direct selection for kanamycin resistance is not practical for low levels of expression and can lead to enrichment for mutated (rearranged) plasmids. To avoid these problems, DNA fragments consisting of the promoter-less kanamycin resistance linked to a second resistance determinant (thiostrepton or viomycin resistance) have been constructed. These fragments, called "Km^R-cartridges", can be conveniently excised from E. coli vectors (which cannot replicate in Streptomyces) and inserted into non-essential regions of Streptomyces plasmids. Thiostrepton or viomycin resistance can be used to select for hybrid plasmids and the level of kanamycin resistance subsequently determined. The Km^R-cartridges have been used to probe for transcriptional activity in the Streptomyces plasmid pIJ101.

STRUCTURAL EVOLUTION OF SELECTED GENE SEGMENTS OF Col E2 AND E3 PLASMIDS

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The DNA sequences encompassing the middle portions and the carboxyl terminal ends of the colicin E2 and E3 polypeptides encoded in plasmids ColE2-P9 and ColE3-CA38 respectively have been determined. Despite the differing catalytic function, the colicin E2-DNAase and the colicin E3-RNAase domains were found to contain several conserved amino acid residues. Moreover, for colicin E3, the amino acid residues which may be involved in catalysis or binding are similar to those of other microbial RNAases. Accordingly, we propose an evolutionary link between the colicin nuclease domains and the various microbial RNAases.

A portion of the receptor-binding domain of both colicins was also identified based on the occurrence of identical sequences as these two colicins share a common btu B receptor on sensitive *E. coli* cells.

We have also determined the sequences of the structural genes of the immunity proteins for colicins E2 and E3. A comparison of the nucleotide and amino acid sequences of these proteins reveals little homology. However, the sequence for the immunity protein produced by plasmid $ColE2_{imm}$-K317 differs from that of ColE2-P9 by only one amino acid change. The single amino acid change may account for the 'partial' immunity protection to colicin E2-P9.

Two regions of deletion in $ColE2_{imm}$-K317 have also been localized, one of which corresponds to a region in ColE2-P9 or ColE3-CA38 where a potential hairpin structure may be found. The structures and evolutionary perspectives of another set of genes, designated hic, whose products are required for the release of colicin from the various producing strains will also be presented.

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PLASMIDS FROM BORDETELLA BRONCHISEPTICA

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Bordetella bronchiseptica has been implicated as a causative agent in the pig disease, atrophic rhinitis. Several possible virulence determinants have been described, though their importance in pathogenesis is not known. Plasmids have been found in some isolates, but their role has not been investigated.

We have examined 14 stains of B. bronchiseptica isolated from pig herds in the U.K. and found plasmids in 7 of these. The plasmids found show considerable homology both by restriction analysis and hybridisation, and we have mapped these regions. Some of the strains had previously been examined for virulence, and we found that virulence did not relate to the presence or absence of plasmids. Six non-pig strains were studied, but none contained plasmids. Strains without plasmids were examined for the presence of plasmid sequences in the genomic DNA.

Phase variation, in which all the virulence- associated factors are not made, has been described in Bordetella. Phase variation was observed in strains both with and without plasmids and no detectable alteration of the plasmids was found.

STRUCTURAL AND FUNCTIONAL STUDIES ON SMALL CRYPTIC PLASMIDS FROM *BACILLUS THURINGIENSIS* H-1

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Except for some of the large plasmids, which in several strains have been shown to contain the δ-endotoxin (crystal) gene, little is known about the structure or the function of *Bacillus thuringiensis* (Bt) plasmids. As a first step towards tackling that problem, we focused our attention on three small plasmids (in the 10 kb range) commonly found in several Bt strains. We used the H1.1 strain as a model system (*Bacillus thuringiensis* var. *thuringiensis* serotype H1, kindly provided by H. de Barjac), harboring the following cryptic plasmids : pBT1 (8.5 kb), pBT2 (9.5 kb) and pBT3 (10.5 kb). We performed experiments dealing with the structure of these plasmids (restriction maps), their function (phenotypic analysis of cured strains, expression into *E. coli* minicells of cloned plasmids) and their distribution among several Bt strains (Southern blots). The following information has been gained from our work :

1. pBT2 and pBT3 have been cloned in *E. coli via* pBR322, and some of their genes have been expressed in this host.

2. pBT1 seems to be "unclonable" in *E. coli* using *Sal*I. This could be due to a lethal effect resulting from the expression of some of its genes.

3. The three small plasmids found in the H1.1 serotype are distinct DNA molecules bearing no strong sequence homology. These sequences are widely distributed among various strains belonging to different serotypes.

4. Whereas pBT1 and pBT3 seem to be well conserved both in size and sequence among the various strains studied, pBT2 appears as a more "labile" plasmid whose sequences can be found on plasmids widely diverging in size. The fact that pBT2 is easy to cure without any significant phenotypic change is another interesting observation with respect to the cryptic nature of that plasmid.

DNA SEQUENCE ANALYSIS OF THE FINO REGION FROM PLASMID R100

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It was observed more than 20 years ago that the transfer of F-like plasmids is inhibited to about 0.1-1% that of the naturally derepressed plasmid F. The mechanism of this fertility inhibition (or fin) is not known. It has been proposed that Fin involves two structural genes finO and finP, whose products interfere with the transcription of the transfer operon's positive control gene, traJ.

Towards our goal of understanding the Fin mechanism at the molecular level, we have cloned and sequenced the finO gene of the F-like antibiotic resistance plasmid R100. This gene is located near R100 kb coordinate 81.3, approximately 30 kb away from finP and at the opposite end of the transfer operon.

The DNA sequence shows one long open reading frame of 558 nucleotides, containing several other possible start codons within the frame. We have isolated and analyzed several frame shift-generating deletions around an XmaI site located 120 nucleotides inside the start of the long open frame. Since some of these deletion mutants are still FinO$^+$, we conclude that, if finO does encode a protein, the structural gene is smaller than the 558 nucleotide maximum size mentioned above. However, other of these mutants were FinO$^-$ and we assume that some aspect controlling finO expression is involved.

An interesting observation was of two regions of strong homology with the λatt sequence (10 and 11 bases, respectively, homologous to the 15 base pairs of λatt). This finding explains the high incidence of λ integration into R100 finO.

HOMOLOGY OF PLASMID DNA TO THE STREPTOMYCES SCABIES CHROMOSOME

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Nine separate strains of Streptomyces scabies have been isolated from potato scab lesions on tubers from Florida, North Dakota and Northern Minnesota. Agarose gel electrophoresis of EcoR1 digested chromosomal DNA from these strains revealed the presence of DNA fragments which appear amplified. The lengths of the amplified bands differ between strains.

One of the S. scabies isolates contains a 64 kilobase plasmid designated pRB1, which we have purified and partially characterized with restriction endonucleases. The pRB1 plasmid has been used as a probe in Southern hybridizations against the chromosomal DNA from its host strain. We found DNA fragments in the chromosomal sample that hybridize to and co-migrate with pRB1 which had been cleaved with the same endonuclease. DNA fragments from the chromosomal sample that hybridize to pRB1 but do not co-migrate with plasmid bands were also observed; the lengths and numbers of these chromosomal bands that are homologous to pRB1 differ depending upon the endonuclease used for cleavage. These results suggest that all or part of pRB1 is integrated into the chromosome. The varying intensities of the bands in the Southern blots indicate that there may be amplification of integrated plasmid sequences.

The pRB1 plasmid was also used as a probe in a Southern hybridization against chromosomal DNA from the other S. scabies isolates. Homology was observed between pRB1 and these other chromosomal samples; the lengths and numbers of homologous bands were different between the chromosomal DNAs tested.

ANALYSIS OF HETERODUPLEX MOLECULES BETWEEN PLASMID p15B AND PHAGES P1 and P7 DNA

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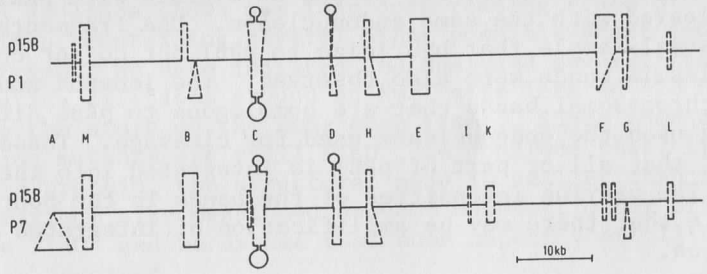
Plasmid p15B isolated from E. coli 15T⁻ and the prophage P1 are closely related: both code for a type III DNA restriction and modification system (1,2), they can recombine with each other (1,3) and they share DNA homology (4). We have determined by electron microscopy the size and location of homologous and non-homologous segments in heteroduplex molecules between p15B and P1 as well as between p15B and the P1-related phage P7 (5,6). The results, summarized in the drawing below, indicate that the genomes share about 80% homology. p15B has substitutions in addition to the regions A-G which are also different between P1 and P7 (5), notably p15B contains two stem-loop structures.



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QUANTITATION OF REGULATORY RNAs - THE pT181 COUNTERTRANSCRIPTS

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The Staphylococcus aureus tetracycline resistance plasmid, pT181, is a plasmid whose rate of replication in indirectly regulated by the intracellular concentration of two small RNA species. It has previously been determined that RepC is a plasmid encoded protein required for pT181 replication and that two small RNA species complementary to the RepC mRNA leader (RNA I of 85 bases and RNA II of 140 bases) are negative effectors of pT181 replication. RNA I and RNA II have a common start within the RepC mRNA leader. When the coding sequence for these small RNAs is cloned onto a carrier plasmid the resultant plasmid inhibits pT181 replication in vivo. This is termed Inc3A incompatibility. Copy mutants of pT181 have been isolated, sequenced, and classed as either dominant (resistant to Inc3A incompatibility) or recessive (sensitive to Inc3A). Utilizing a method of Northern Blot analysis, RNA from whole cell lysates was separated on formaldehyde-agarose gels and the relative amounts for RNA I and RNA II were determined. For the point mutants tested, dominant copy mutants make the small RNAs in a manner proportional to their increase in copy number (gene dosage). One recessive mutant maps in the -10 promoter sequence for RNA I and RNA II, and another in the -35 region. Strains carrying these high copy mutant plasmids have the small RNAs at the same intracellular concentration as the wild-type and therefore make less of RNA I and RNA II per plasmid copy. Other recessive mutants appear to be defective in the termination of either RNA I or RNA II, thus indicating that both molecules are involved in the negative control of pT181 replication.

SEQUENCE ANALYSIS OF Tn917

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Tn917 (Em) is a 5.0 kb transposon identified in Streptococcus faecalis that has been shown to undergo enhanced transposition when exposed to inducing concentrations of erythromycin. DNA sequencing has revealed terminal inverted repeats which are 38 base pairs in size, but are not completely identical. They are highly homologous to the inverted repeats of Tn551 (Khan, S., and R. Novick. Plasmid 4:148-154 1980) and Em-resistance transposon isolated from Staphylococcus aureus; both show homolgy with the inverted repeats of Tn3.

Sequences closely resembling the MLS determinant reported by Weisblum, et al., (J. Bacteriol. 154:1252-1262 1983) for pAM77 (a 6.75 kb plasmid originally identified in Streptococcus sanguis) have been found near one end of the transposon. In addition, the resistance determinant is flanked by short open reading frames similar to sequences observed in pAM77. The sequence 5' to the MLS resistance determinant is believed to represent a "leader sequence" involved in attenuative translational control of the synthesis of a ribosome methylating enzyme. Although a function for the sequence on the 3' end is unknown, a possible attenuational role in Em-induced transposition is conceivable. The latter sequence contains a nearly complete copy of a 38 base pair terminal repeat in the same orientation as that located on the opposite side of the resistance determinant. Closely adjacent to this repeat is a 20 base pair sequence resembling the res site of Tn3. A similar sequence is not present on pAM77; however, the equivalent region contains two copies resembling the "repeat sequence". They are present in opposite orientation and overlap each other by 16 base pairs. It is conceivable that the evolution of pAM77 involved a Tn917 which acquired a deletion.

Evidence that the Clindamycin-Erythromycin Resistance Gene of the *Bacteroides* Plasmid pBF4 is on a Transposable Element

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The conjugative Bacteroides plasmid, pBF4 (pIP410), carries a gene for clindamycin-erythromycin resistance which is expressed in Bacteroides (Privitera et al., J. Inf. Dis. 139:97; Welch et al., Plasmid 2:261). pBF4 also carries a tetracycline resistance gene which is expressed in E. coli but not in Bacteroides (Guiney et al., Plasmid, in press). We have constructed a chimeric plasmid, pE5-2, which contains the E. coli plasmid RSF1010, plus a cryptic Bacteroides plasmid (pB8-51) and a 3.8 Kb EcoRI fragment from pBF4. pE5-2 confers tetracycline resistance on E. coli. It is mobilized by the broad host range plasmid R751 from E. coli to Bacteroides thetaiotaomicron or Bacteroides uniformis with a frequency of 10^{-6}. pE5-2 was the only plasmid which was recovered from Bacteroides transconjugants whereas 98% of the E. coli transconjugants, contained both R751 and pE5-2. The Bacteroides transconjugants were resistant to clindamycin-erythromycin. This is the first direct evidence that these resistances are located within the EcoRI-D fragment of pBF4. We have also constructed a chimeric plasmid, pSS2, which contains a much larger portion of pBF4 (33 Kb). pSS2, unlike pE5-2, was not stable in E. coli. Mobilization of pSS2 by R751 from E. coli to E. coli, was associated with a variety of rearrangments. In some cases a segment of pBF4 was inserted into R751. This segment included the EcoRI-D segment which confers tetracycline resistance in E. coli and clindamycin-erythromycin resistance in Bacteroides. In two cases, the same segment appeared to have inserted in the E. coli chromosome. Thus it appears that there is a transposable element which contains the EcoRI-D fragment of pBF4. We did not detect any transfer of pSS2 from E. coli to Bacteroides.

CONJUGATIVE MAPPING OF *PSEUDOMONAS PUTIDA* USING XYL-K

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Pseudomonas putida has been used extensively for biochemical studies because of its metabolic versatility, however, genetic studies have been hampered by the lack of a conjugative system. Mylroie *et al*. (1977) reported the use of the recombinant plasmid-sex factor, XYL-K for mapping the *P. putida* chromosome and Dean and Morgan (1983) constructed the high frequency recombination plasmid R91-5::Tn501 to map and to prove the circularity of the *P. putida* chromosome. We are interested in the biochemistry and genetics of the three keto acid dehydrogenases of *P. putida*, branched chain keto acid dehydrogenase (bckad), pyruvate (pdh) and 2-ketoglutarate (kgdh) dehydrogenases. In addition, *P. putida* produces two distinct lipoamide dehydrogenases, LPD-val and LPD-glc. LPD-val is specifically required as the E3 component of bckad; LPD-glc is required as the E3 component of kgdh, probably pdh and for glycine oxidation. We have isolated mutants of *P. putida* in bckad, pdh, kgdh, LPD-val and LPD-glc as well as auxotrophic and catabolic mutants by mutagenesis with nitrosoguanidine or with plasmid pRKTV14. Conjugation was effected with XYL-K using strain AC143 as the donor. Filter matings were performed as described by Mylroie *et al*. (1977) except that streptomycin was not always used in counterselection. Times of entry for pdh and kgdh were 22 and 10 min respectively. Mutants of *P. putida* deficient in all three subunits of bckad have been obtained (E1, E2 and LPD-val) and all map at about 35 minutes. There is good agreement with map locations of Mylroie *et al*. for *put* and *hut* and the data are consistent with a single point of origin.

GENETIC ORGANIZATION AND FUNCTION OF THE PLASMID pKM101

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The *incN* plasmid pKM101 was derived from its clinically isolated parent R46 by an in vivo deletion of 14 kb which removed genes coding for drug and metal resistances. pKM101 has been extensively studied because it increases the susceptibility of cells to UV and chemical mutagenesis by coding for the *mucA* and *mucB* genes which analogs of the chromosomally encoded *umuD* and *umuC* genes. It has played a major role in the success of the Ames *Salmonella* strains used for detecting mutagens.

The conjugal transfer system of pKM101 consists of three regions, TRA I, TRA II, and TRA III, each separated from each other by a region not absolutely required for transfer. All the genes in the first two regions are required for sensitivity to donor-specific phage. The plasmid's origin of transfer has been localized to a region at the far end of TRA III. Using two methods we have identified eleven complementation groups needed for transfer. The TRA I and TRA II regions are separated from each other by *eex*, a gene mediating entry exclusion. The TRA II and TRA III regions are separated from each other by *nuc*, a gene coding for a periplasmically located endonuclease, and by *fip*, a gene whose product inhibits the fertility of P group plasmids.

Two pKM101-coded loci, *kilA* and *kilB*, have been identified which elaborate gene products that are potentially lethal to the bacterial cell. The lethal effect of each of these gene products is inhibited by two other plasmid-coded loci, *korA* and *korB*, both of which are required to control the lethality of either *kil* gene. In the presence of *korA* and *korB* both *kil* genes have other phenotypes: *kilB* is necessary for conjugal transfer, while *kilA* is responsible for the small colony morphology on defined media that is characteristic of pKM101-containing strains.

PLASMIDS OF BACTEROIDES FRAGILIS

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A plasmid screenig procedure was developed for lysozyme resistent Bacteroides. 24 strains of B. fragilis, subsp. fragilis, isolated from suppurative infections, were analysed for their plasmid content, 9 strains contained 1 to 3 plasmids. Plasmids could be divided into 5 classes of 2.8, 3.5, 3.6, 4.2 and 19 MDa size. From multiple occurence we conclude, that the small plasmids belong to different compatibility groups. The small plasmids were also observed in strains, not belonging to subsp. fragilis. By Southern blotting it was shown, that homology exists only between plasmids of the same size. Plasmids were digested with 17 restriction endonucleases, plasmids of the same size gave identical fragmentation patterns. Restriction maps were constructed from partial and double digestion. The 19 MDa plasmid showed no transposon like structures by homoduplex analysis. Presently no function can be assigned to the plasmids. No dependence was observed between plasmid content and bacteriocin and hemolysin production. Resistance against 9 antibiotics (penicillin, clindamycin, tetracyclin, chloramphenicol, erythromycin, ampicillin, kanamycin, streptomycin and rifampicin) showed no correlation to the occurence of particular plasmids.

SECONDARY STRUCTURE PREDICTIONS FOR RNA TRANSCRIPTS FROM THE NR1 REPLICATION CONTROL REGION

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The repA1 protein of the IncFII plasmid NR1 is required for initiation of DNA replication at the plasmid origin. Synthesis of repA1 is negatively regulated at both the transcriptional and translational levels. Translation of the repA1 mRNA is inhibited by a small, complementary RNA transcribed from the opposite strand. The inhibitor RNA is synthesized in a 20-fold excess, and a majority of the repA1 messengers pair with the inhibitor RNA. The predicted secondary structure of repA1 mRNA which has paired with the inhibitor RNA has the start codon and ribosome recognition sequence for repA1 translation paired in an intramolecular double-stranded structure. This should prevent ribosome binding and inhibit repA1 synthesis. For the rare repA1 mRNA which escapes pairing with the inhibitor RNA, the predicted secondary structure has both the start codon and ribosome recognition sequence in single-stranded regions. This should allow ribosome binding and repA1 synthesis, which will then cause initiation of DNA replication at the plasmid origin. The choice between these alternate secondary structures is the "switch" which regulates initiation NR1 DNA replication.

AUTOGENOUS REGULATION OF THE π STRUCTURAL GENE OF PLASMID R6K

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It has been proposed that binding of the R6K π initiation protein to the seven 22 bp repeats in the γ-origin of replication and to the eighth repeat near the pir (π structural gene) promoter are required for initiation of plasmid R6K replication and autoregulation of pir gene expression, respectively (1). Evidence has been obtained for the autogeneous regulation of the pir gene (2) and the specific binding of a β-galactosidase-π fusion protein to the tandem seven direct repeats and the 8th repeat (3). In the present study a plasmid was constructed that placed the β-galactosidase gene under control of the pir promoter. Greater than 95% repression of β-galactosidase expression from this plasmid was observed in E. coli cells when the complete pir gene is present in trans. Alterations in the N-terminus sequence for the π protein substantially reduces its autoregulatory activity. Purified π protein also was shown in vitro to specifically repress transcription from the pir promoter. Binding sites on the pir promoter for the π protein and E. coli RNA polymerase were delineated by DNaseI protection experiments. Purified π protein and RNA polymerase bind to the operator-promoter region of the pir gene which includes the 8th repeat. Since only a portion of π binding site is shared with the RNA polymerase binding site, the pir operator-promoter region appears to be unusual. In addition, purified π protein not only prevents binding of RNA polymerase but it displaces bound RNA polymerase from the operator-promoter region of the pir gene.

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traT GENE AND ITS SURFACE EXCLUSION CHARACTERISTICS

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The ColV plasmid was given a genetic marker by insertion of the kanamycin resistance transposon Tn903 to yield plasmid pWS12; this derivative was found to possess all normal colV properties. Since Tn903 has a tendency to insert near an origin of replication, we used this fact in cloning restriction fragments carrying the replicons of ColV. Restriction and ligation of the 130 kb pWS12 yielded the self-replicating fragments pWS15 (using *Eco*RI) and pWS16 (using *Bam*HI), which were 32 kb and 75 kb in size, respectively. The plasmid pWS12 and its deletion derivatives pWS15 and pWS16 carry the surface exclusion (*SEx*) genes *tra*T and *tra*S of the ColV2-K94 transfer region. The parent plasmid pWS12 and its self-transmissible derivative pWS16 showed *SEx* towards the plasmids of specificity group II. Interestingly, pWS15, Tra$^-$ derivative of pWS12, surface excluded plasmids of *SEx* groups I and II. However, pWS15 and pWS12 produced a similar amount of *tra*T protein in the outer membranes of host cells. The molecular weights of the *tra*T proteins produced by pWS12 and pWS15 were identical as determined by SDS-PAGE. These proteins were also immunologically cross-reactive. Thus it appears that plasmid gene(s) within coordinates 32V to 75V outside the *tra*T and *tra*S loci regulate expression of specificity group I and II in this system.

MOLECULAR DETAILS OF THE GENES FINO AND FINP WHICH CONTROL THE INFECTIOUS TRANSFER OF THE ANTIBIOTIC RESISTANCE PLASMID R100

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The finO and finP genes of R100 are two of three plasmid genes that regulate fertility of R100. They both act in trans but their gene products are not yet unequivocally identified. To get more information about finO, finP and tra, all of the EcoRI fragments that comprise the R100 tra operon have been cloned into pBR322 or pBR325 and used to form a detailed restriction map of tra. From this information, finO, finP, traJ, oriT, and traOYA of R100 have been isolated on small fragments and subcloned individually into pHP34. The finO gene resided entirely on a 735bp fragment whose ends map at R100 coordinates 81.11 and 81.84. The finP gene resided entirely on a 700bp fragment whose ends mapped at R100 coordinates 46.52 and 47.22. S1 nuclease mapping with these isolated fragments was unsuccessful. Runoff transcripts from the finO fragment showed one or two RNA's of 60-80bp encoded in the far right end. No transcripts were found from the finP fragment. The isolated finO and finP genes were used in hybridization experiments to probe the tra genes for stringent and weak homologies. None were found between these genes or with any other part of the R100 molecule. Chromosomal probing showed no hybridization. Specific hybrids were formed at low stringency between the finP probe and most of the fragments of a commercially available DNA molecule weight standard. The finO probe on the other hand hybridized specifically with only two of the bands of that standard, one uniquely. The structure of the standard being unavailable, digests of pBR322 were probed. The finO fragment was found to hybridize at moderate stringency to pBR322 in the amp gene and to the region between 1800 and 2000bp on the pBR322 map.

DISSECTION OF THE TRANSFER REGION TraI OF PLASMID RP4 — EXPRESSION AND MOLECULAR CHARACTERIZATION OF GENE PRODUCTS

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The conjugative broad-host-range Inc Pα plasmid RP4 contains three unlinked transfer regions (Tra) which are essential for self-transmissibility of the plasmid from donor to recipient cells. One of the regions, TraI, is located between Km^r and trfB; it includes the origin of transfer (oriT) where initial events of transfer DNA replication requiring plasmid-specified proteins must occur. Five putative promoter sites have been localized within TraI by RNA polymerase binding studies. Gene products of this plasmid portion are being studied. Transcription of a major part of TraI appears to proceed in opposite direction to that of Km^r. Efficient expression of five polypeptides requires only one of the promoters suggesting the synthesis of a polycistronic messenger. The molecular masses of the proteins determined on denaturing gels are 16.5, 68, 77, 80 and 118 kDa. The primase gene (pri, 3.2kb) specifies two proteins of 118 and 80 kDa. As shown by deletion analysis of recombinant Pri^+-plasmids and immunological studies, the primase polypeptides represent two separate translation products which arise from an in-phase overlapping gene arrangement. Both these polypeptides mediate initiation of complementary strand synthesis on single-stranded phage DNA *in vitro* by synthesis of RNA primers. Two genes specifying polypeptides of 26 and 52 kDa map in close vicinity to oriT as demonstrated with a minicell system. The gene for the 26 kDa protein has been localized adjacent to the oriT sequence. Molecular cloning of the structural gene into an expression vector facilitated overproduction and purification. Subcloning of the oriT region has revealed that the fully functional origin comprises ∿320bp. The sequence consists of two regions. One includes the putative nic site on a stretch of 112 bp (Yakobson and Guiney,1983, Proc.Nat.Acad. Sci.USA 80,3595). The second region, which contains a promoter sequence and probably additional recognition sites for transfer proteins, is necessary to retain a high mobilization frequency of oriT recombinant plasmids. The location of the promoter coincides with one of the sites found by RNA polymerase binding studies.

FORMATION OF ΔtraF' PLASMIDS: SITE-SPECIFIC RECOMBINATION NEAR oriT

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Mating between Hfr strains and $recA^-$ recipients with selection for proximal Hfr markers produces ΔtraF' plasmids, which contain the proximal region of F up to one junction with chromosomal DNA. These plasmids are nonconjugative, and the entire tra operon is deleted. Restriction analysis indicates that the process can be site-specific, with the novel joints mapping near oriT.

BglII fragments containing novel joints from Δtra F $proA^+$ or Δtra F $purE^+$ plasmids were cloned into a mob^- vector pED961, and the resulting plasmids were tested for mobilization from $recA^-$ strains by the F derivative pOX38. Plasmids containing oriT of F were mobilized at frequencies of 10^{-1}-10^{-2} per input donor, but plasmids containing novel joints from the ΔtraF' plasmids were not (frequencies < 10^{-7}-10^{-8}), indicating a cis-dominant defect.

The regions containing the novel joints, the corresponding region of F, and the relevant chromosomal segment near purE were sequenced. The nucleotide sequence of the novel joint region was identical for 4 independent Δtra F $purE^+$ isolates. This also was true for the Δtra F $proA^+$ plasmids. The F DNA joined bacterial DNA 135-140 bp clockwise of the BglII site at the 66.6 kb F map position in both plasmid types. The chromosomal sequence involved in Δtra F $purE^+$ formation (F homologies are underlined) is

$$\begin{matrix} 5' & A & C & A & C & C & A & C & A & C & G & 3' \\ 3' & \underline{T} & \underline{G} & \underline{T} & \underline{G} & \underline{G} & T & \underline{G} & \underline{T} & \underline{G} & \underline{C} & 5' \end{matrix}$$

One Δtra F $purE^+$ terminated at the same position in F but at a different chromosomal site.

During ΔtraF' formation, recombination events have occurred in the oriT region at one defined sequence on F. This sequence may include the site of nicking in oriT.

BACK TRANSFER : A PROPERTY OF SOME BROAD HOST RANGE PLASMIDS

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Back transfer would be defined as a plasmid-mediated transfer of genes mediated in the plasmid bearing strain itself. Three lines of evidence supporting such a definition are here reported :

1) Homologous matings mediated by IncP1 plasmid pULB113(RP4::miniMu)
In such matings, an excess of recombinants carrying all the markers of the plasmid bearing strain but the counterselected one, was regularly observed : in a mating between S. typhimurium strains, the pULB113 bearing strain carrying seven markers (including Δ trp BA196) dispersed on the chromosome was mated with a single marked Δ hisBHA recipient. His^+ Trp^+ recombinants were selected and up to 95% of recombinants carried the markers of the plasmid bearing strain (metA22, metE331, ilv452, xyl 404, rpsL120, galE493) and were thus believed to be Trp^+ backtransconjugants instead of the expected His^+ direct transconjugants. In P. fluorescens 6.2, the Tra^- Mob^- IncQ plasmid pKT261 was introduced by transformation to mark the recipient strain and allowed to discriminate direct (normal) and back transconjugants.

2) Heterospecific matings mediated by RP4::miniMu
In matings between pULB113 bearing P. fluorescens and the unrelated strain Alcaligenes eutrophus CH34, R-primes were found at similar frequencies in both parents. In P. fluorescens the back R-primes carried up to 70 kb of genetic material coming from A. eutrophus.

3) Mobilization of plasmids poorly or non self transferable
Back mobilization of the Tra^- IncQ plasmid R300B was mediated in E. coli by plasmids RP4 and pUZ8 (IncP1), pULG14 (IncN) and R69.2 (IncM) at a frequency of 10^{-2} to 10^{-5}. Back mobilization of R300B was not induced by plasmids pSa and R339 (IncW), pIP113 and N3 (IncN). Plasmid pULG14 (Erwinia uredovora) attracted pMOL28 (165 kb; resistance to nickel and cobalt) from A. eutrophus CH34. Similarly, IncP1 plasmid pJP1 (catabolism of 2-4D) attracted in its resident strain A. eutrophus JMP134, pMOL28 or pMOL30 (238 kb ; resistances to Zn^{++}, Cd^{++} and Co^{++}) from A. eutrophus CH34. The back transconjugants resulted mainly in stable cointegrates in JMP134.

GENERALIZED TRANSDUCTION OF PLASMID REPLICONS BY BATERIOPHAGE P22 IN SALMONELLA TYPHIMURIUM

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Plasmid pBR322 and various derivatives with inserts of the trp region of the S. typhimurium chromosome can be transferred between strains of S. typhimurium by generalized transduction with bacteriophage P22. The chimeric plasmids are transduced about 20-fold more efficiently than pBR322. $RecA^-$ donors as well as $RecA^-$ recipients are capable of plasmid transduction; however, the frequency is 5- to 10-fold higher with $RecA^+$ strains.

Gene 3 mutants of P22 which exhibit high frequency transduction of chromosomal markers show a corresponding increase in plasmid transducibility over that of wild type phage. Lysates of Erf^- P22 grown on $RecA^-$ donors retain the ability to transduce plasmids even though the titer of plaque forming units is severely reduced.

Plasmid DNA is packaged into transducing particles as a head to tail linear concatemer. The possibility that this DNA arises by the initiation of rolling circle replication of the plasmid upon infection of the donor strain by P22 is currently being examined. In this regard, Nic^- derivatives of pBR322 have been found to be transduced as efficiently as pBR322.

Both monomeric and a variety of multimeric plasmid replicons have been found to be established in individual transductant clones, apparently by a process of recircularization that is not necessarily RecA-dependent.

Besides serving as a convenient method for the transfer of plasmids between strains of S. typhimurium, plasmid transduction promises to be a model system for the analysis of the mechanism of formation of generalized transducing particles in P22 infections.

ROLE OF TRANSCRIPTION IN CLO DF13 PLASMID MOBILIZATION

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The non-conjugative, bacteriocinogenic plasmid Clo DF13 (m.w. 6 Md), can be mobilized efficiently in the presence of a conjugative plasmid, such as F or R. The mobilization process requires both gene products of the conjugative plasmid and Clo DF13 encoded proteins as well as a cis-acting Clo DF13 region, termed bom (basis of mobility). This bom region is located withi a 264 bp fragment around the unique HpaI site. Comparison with the corresponding Col E1 and pBR322 sequences showed similarities with respect to the secondary structure. With respect to a possible relationship between the origin of vegetative replication (oriV) and the origin of transfer (oriT) we found that neither distance nor orientation of the Clo DF13 bom region with respect to oriV had any significant influence or the mobilization frequency. Surprisingly, after cloning of the 264 HpaII fragment in a Bom^- vector, restoration of the Bom^+ phenotype was only observed in one orientation. From these observations and from our sequence data we suggest that transcription into this cloned bom fragment is essential for effective mobilization of the plasmid.

TRANSFORMATION OF CLOSTRIDIUM PERFRINGENS WITH CHIMERIC PLASMID DNAS

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L-phase variants, or autoplasts, of C. perfringens may be transformed with plasmid DNA in the presence of polyethylene glycol. L-phase variants are stable replicating protoplasts generated by growth of normal cells with penicillin G in osmotically protected medium. Approximately 1,000 to 10,000 transformants can be obtained per ug of C. perfringens DNA used; this is a frequency of 1/10,000 to 1/100,000 transformants/cell/ug DNA. C. perfringens will form autoplasts (self-generated protoplasts) if cultured in osmotically protective medium without penicillin G. These cells can be transformed at frequencies similar to those for L-phase variants. The cell wall of these transformants may be regenerated by growth in liquid medium containing 25% gelatin. The plasmid DNAs used to transform C. perfringens are chimerae of E. coli plasmid pBR322 and whole, cryptic, small C. perfringens plasmids cloned to it for replicon function. In addition, these plasmids contain 4 kb of C. perfringens plasmid DNA coding for resistance to tetracycline. This tetracycline resistance function is competent in both E. coli and C. perfringens.

IDENTIFICATION OF MULTIPLE SURFACE ANTIGENS ASSOCIATED WITH SEX PHEROMONE RESPONSE OF STREPTOCOCCUS FAECALIS.

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High molecular weight surface antigens of S. faecalis obtained by ammonium sulfate precipitation of culture supernatants and identified in Western blots of SDS-polyacrylamide gels, have been correlated with the aggregation response of donor cells to sex pheromone ("clumping inducing agent," CIA).

CIA induced cells carrying the conjugative plasmid pCF10 produced a greater amount of a surface antigenic doublet having a subunit molecular weight in the 130,000 range than did uninduced cells or isogenic plasmid free cells. Additional unique bands having molecular weights of approximately 73,000, 134,000 and 138,000 were observed in CIA induced cells. A unique 160 kilodalton antigen was also produced by an isogenic constitutively clumpy mutant.

The 130 kilodalton antigenic complex increased in concentration with time after exposure of donor cells to CIA. Gel filtration studies indicated that this antigen exists in the native state at least as a dimer and perhaps as large as a hexameric complex. This component was susceptible to digestion by proteinase K and was not reactive with either concanavalin A or wheat germ agglutinin. The antigenicity of this component was not destroyed by treatment of blots with trypsin, chymotrypsin or papain prior to development with antibody.

USE OF *E. COLI* GALACTOKINASE TO PROBE FOR *STREPTOMYCES* PROMOTERS

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Plasmids designed to identify and characterize *Streptomyces* transcriptional regulatory elements have been constructed using the *Escherichia coli* galactokinase gene. The plasmids are shuttle vectors containing the pBR322 *E. coli* replicon and the pIJ350 *Streptomyces* plasmid, permitting rapid assembly and characterization of plasmid constructs in *E. coli* followed by transformation into *Streptomyces* for functional analysis. The *galK* gene is preceded by a series of unique cloning sites and a fragment, containing the tR1 transcription terminator of phage lambda, which prevents readthrough transcription into *galK*. A DNA fragment preceding the *Streptomyces* gene which encodes a β-galactosidase-like enzyme and the promoter fragment from the *S. fradiae aph* gene were cloned into a promoter probe plasmid and these fragments stimulated the synthesis of galactokinase in *Streptomyces*. The 5' end of the β-gal-galactokinase transcript was identified by S1 mapping and found to be near an AT rich region, also identified by S1 mapping of the chromosomally-encoded β-gal transcript. The level of galactokinase produced when controlled by the β-gal promoter was 10% of the total cellular protein. This promoter should prove useful in the construction of *Streptomyces* gene expression vectors.

PLASMID CLONING VEHICLES - NEW EXPRESSION VECTORS BASED ON THE P_L PROMOTER OF COLIPHAGE λ; EFFICIENT SYNTHESIS OF HUMAN INTERFERONS AND INTERLEUKIN 2

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We have earlier described a plasmid expression system based on the inducible leftward promoter of coliphage λ (1). New derivatives have been constructed which incorporate a prokaryotic ribosome binding site and an easily accessible ATG initiator codon. The ATG codon is part of a NcoI site (CCATGG). Following NcoI cleavage and filling-in of the sticky ends, the ATG codon can be readily joined to any blunted coding region. Because of its much greater fidelity, the filling-in reaction is more advantageous than the previously used S1 nuclease in exposing the ATG codon (2). Ribosome binding sites were obtained from the replicase gene of phage MS2, the *ner* gene of phage Mu, or a modified attenuator region of the *E. coli trp* operon. In each case, a unique NcoI site was engineered at the initiation codon. Several unique restriction sites are situated further downstream. The vectors are small (2.8 Kb) and their complete nucleotide sequence is known.

We have used these vectors to obtain inducible, high-level synthesis in *E. coli* of human interferons and interleukin 2. β-interferon could be boosted to make up 2% of the total cellular protein. For interleukin 2 and γ-interferon, levels of 5% and 25%, respectively, were obtained.

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INCLUSION OF PLASMIDS INTO LARGE LIPOSOMES: POTENTIAL FOR ELECTRIC FIELD INDUCED FUSION WITH CELLS

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We are interested in transfer of genes from cell-sized, DNA-carrying liposomes into target cells by electric field induced fusion described by Zimmermann[1].

I. Preparation of large liposomes: Liposomes with a diameter up to 100 µm were prepared by swelling lipid multilayer films in a medium of low ionic strength. An equimolar mixture of cholesterol and different natural and synthetic lipids [2] was used.

II. Inclusion of plasmid DNA: Large liposomes were prepared by initial swelling of lipid films with a small volume of aqueous pBR322 solution. For control of the amount of included DNA the liposomes were spun down by centrifugation, resuspended and the solution was than digested with DNase I to eliminate DNA in the outer space. After destruction of the vesicles by adding detergent solution the DNA was directly shown on agarose gel electrophoresis. The inclusion rates were up to 14% of the DNA input.

III. Fusion of empty liposomes with each other or with cells: Intimate contact between liposomes was established by the application of an alternating nonuniform field, resulting in dielectrophoresis and pearl chain formation. The fusion process was initiated by a field pulse of 3-9 $kV.cm^{-1}$ and 20-50 µs duration. The first experiments to realize a fusion of vesicles with Friend cells have been carried out successfully[3].

Fusion experiments with bacteria have thus far been unsuccessful because it is still unclear how to prepare the bacteria before the experiments. Therefore we plan to first study the electric field induced fusion between DNA-carrying liposomes and Saccharomyces cerevisiae cells.

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A NOVEL BACTERIAL-YEAST SHUTTLE VECTOR SYSTEM FOR CLONING AND INVESTIGATING STRUCTURAL OR REGULATORY DNA REGIONS

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We report the construction of several unique cloning vectors composed of bacterial and yeast DNA which allow replication and selection in both organisms. Subsequent facile manipulations transmute these shuttle vectors into Yeast Acentric Ring plasmids, (YARp's). This system has distinct advantages over other shuttle vectors. One important advantage is that the YARp's constructed will be derivatives of the TRP1 RI Circle (YARp1) which contains only the TRP1 coding region and an autonomously replicating sequence, ARS1. YARp1 has been shown to transform yeast at high frequency, to be established at a copy number of 100-200 per cell, and to have high stability as compared to most shuttle plasmids containing non-yeast DNA. Another important property of this system is that YARp1 can be isolated as a protein-associated chromatin complex relatively free from other cellular constituents. Also, various DNA sequences have been inserted into YARp1 resulting in chimeric plasmids retaining properties similar to the parental YARp1, thus establishing its validity as a cloning vector. For example, taking advantage of selection and screening techniques in bacteria, we cloned a galactose-inducible yeast promoter into a unique EcoRI site proximal to the TRP1 coding region in one of the shuttle vectors. Cleavage by one restriction enzyme released the combined yeast fragment which was then recircularized with DNA ligase. The resulting YARp may prove to be a superior plasmid for some structural and functional studies of galactose regulation in yeast.

EXPRESSION OF BOVINE GROWTH HORMONE IN E. COLI: ENHANCED EXPRESSION BY ALTERING SEQUENCES OF THE CODING REGION WITH SYNTHETIC DNA FRAGMENTS

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The DNA sequences required for high level expression of bovine growth hormone genes in E. coli was investigated using temperature-sensitive copy control plasmids as cloning vectors. By changing the 5'-terminal coding region of the bovine growth hormone (BGH) gene with various synthetic DNA fragments, we were able to dramatically alter the level of expression. Some BGH gene constructions gave greater than 35 percent of the total cell protein as methionyl bovine growth hormone (met-BGH), while the original cDNA met-BGH gene gave very low espression (1 percent).

The results showed that minor changes (one or few base changes) in the 5'-terminal coding region of cloned BGH gene can have a profound effect on BGH expression. A possible correlation between the increase in the level of the BGH expression and the destabilization of potential 5'-end mRNA secondary structure in the region of the initiation codon, Shine-Dalgarno sequence, and the NH_2-terminal sequences of the BGH genes is discussed.

EXPRESSION OF THE Escherichia coli XYLOSE ISOMERASE GENE IN Bacillus subtilis USING A Bacillus PROMOTER

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A DNA fragment containing both the E. coli D-xylose isomerase gene (xylA) and D-xylulokinase gene (xylB) has been isolated from an E. coli genomic bank constructed by Clarke and Carbon. The D-xylose isomerase gene was characterized and then separated from the D-xylulokinase gene by a series of subcloning processes. D-Xylose isomerase, the enzyme product of the xylA gene, is responsible for the conversion of D-xylose to D-xylulose, as well as D-glucose to D-fructose. We have subcloned the intact xylA gene into an E. coli and Bacillus shuttle plasmid. The resulting plasmid was used to transform Bacillus. However, enzymatic assay indicated that the intact E. coli xylA gene was not functioning in Bacillus transformants. We believed this was due to the ineffectiveness of the E. coli xylA promoter in Bacillus.

In order to express the xylA gene in Bacillus, we have isolated the gene encoded for the synthesis of β-lactamase (penicillinase), penP, from B. lichenformis. It has been shown that the promoter of penP functions properly in both E. coli and Bacillus. Basically, we are trying to replace the xylA promoter with penP promoter. The promoter region of the E. coli xylA gene was deleted and the resulting xylA structural gene was inserted downstream to the promoter of the penP gene. The resulting hybrid gene (penP-xylA) complements E. coli xylA mutation. Bacillus transformants containing the hybrid gene produced elevated D-xylose isomerase activity. Improvement of the expression of D-xylose isomerase gene in Bacillus is in progress. (Supported by NSF PCM-8305043, and SERI Sub-contract XX-3-03116.)

LOW-, INTERMEDIATE-, AND HIGH-COPY-NUMBER CLONING VECTORS DERIVED FROM THE PSEUDOMONAS PLASMID pVS1

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The Pseudomonas plasmid pVS1 was reduced to a minimal replicon of ca. 3 kb and used to construct gene cloning vehicles. Several non-mobilizable or RP1-mobilizable plasmids were obtained. Each carried two resistance markers for selection and insertional inactivation, respectively. The Mob$^-$ vectors pME290 (Cb Km) and pME291 (Hg Km) could be introduced by transformation and maintained in P. aeruginosa, P. putida, P. fluorescens, P. stutzeri, P. acidovorans, P. mendocina, P. cepacia, and P. syringae.

Since the vector copy number appears to influence critically the stability of recombinant plasmids in P. aeruginosa PAO, we isolated mutants of pME290 (ca. 7 copies) with different copy numbers: pME292 (1-2 copies) and pME294 (ca. 15 copies). The argF gene of P. aeruginosa cloned into these vectors was expressed according to their copy numbers and the recombinant plasmids were stable in P. aeruginosa during argF repression (in arginine medium) and derepression (in glutamate medium). However, under repressive conditions 15 argF copies had a strong inhibitory effect on growth and an argF$^+$ recombinant plasmid derived from the RSF1010 vector pKT240 (≥20 copies) led to severe physiological disturbances and was entirely unstable in P. aeruginosa.

CONSTRUCTION OF AN IMPROVED MAXICELL STRAIN FOR THE IDENTIFICATION OF RECOMBINANT PLASMID ENCODED PROTEINS

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An improved "maxicell" host strain of Escherichia coli [MCL22: trkA405 trkD1 thi rpsL31 Δ(kdp-phr)214 Δ(gal-uvrB) Δ(srl-recA)306 rha], has been constructed to avoid the most common problems blocking identification of plasmid-encoded proteins by the method of Sancar et al. (J. Bacteriol. 137, 692, 1979). (1) Use of deletions, rather than point mutations, to inactivate genes of the DNA repair pathways [photoreactivation (phr), excision-repair (uvrB), and recombination-repair (recA)] prevents inadvertent reversions, and the attendant, unwanted bacterial chromosomal protein synthesis. (2) Absence of nutritional deficiencies allows a more vigorous growth than that shown by previously used strains (45 minute doubling time in Luria broth, and growth in minimal media without amino acid supplement) facilitating establishment of cultures free of slow-growing cells. The strain also shows high efficiency of transformation by plasmid DNA (>10^6 transformants per μg pBR322 DNA). Efficacy of these features is shown in tests to distinguish plasmid-encoded protein products of pBR322.

CLONING VECTORS DERIVED FROM THE LOW COPY *STREPTOMYCES* PLASMID SCP2*: USE OF SUCH A VECTOR IN ISOLATING THE ENTIRE SET OF BIOSYNTHETIC GENES FOR AN ANTIBIOTIC

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SCP2* (31 kb), a variant of the natural plasmid SCP2 of *Streptomyces coelicolor* A3(2), exists in 1-2 copies per host chromosome. By *in vitro* deletion of non-essential DNA, and addition of one or more marker genes, we have derived a series of useful cloning vectors. As well as sequences essential for replication, they all retain a "partition" function needed for stable plasmid inheritance. They include pIJ903 with unique cloning sites for several enzymes: *Bam*HI, *Eco*RI, *Hin*dIII, etc; pIJ916, with sites for *Bgl*II, *Sst*I, etc; pIJ922, with sites for *Bam*HI, *Pst*I, etc; and pIJ940, with sites for *Bam*HI, *Bgl*II, *Pst*I, etc. All four vectors carry the *tsr* gene for thiostrepton resistance and pIJ940 carries hygromycin resistance in addition. pIJ903, pIJ922, and pIJ940 are transmissible by conjugation, while pIJ916 is non-transmissible.

These vectors are very suitable for the stable cloning at low copy number of large segments of *Streptomyces* DNA. For example, pIJ922 (24.5 kb) has been used to clone DNA from the wild-type *S. coelicolor* into mutants blocked in actinorhodin biosynthesis (*act*). Size-fractionated donor chromosomal DNA (15-30 kb), produced by partial digestion with MboI, was inserted into the *Bam*HI site of pIJ922 and introduced by transformation into protoplasts of an *act* mutant. Two Act$^+$ clones were obtained, with overlapping inserts of 16 kb and 34 kb. By *in vitro* reconstruction, a single clone (pIJ2303 with an insert of 32.5 kb) was obtained which complemented all known classes of *act* mutants. It also promoted actinorhodin synthesis in an unrelated species, *Streptomyces parvulus*. It therefore appears to carry the entire set of genetic information needed to make this polyketide antibiotic from acetate. Even plasmids as large as PIJ2303 can transform *Streptomyces* protoplasts at good molar efficiency.

λPL-COLE1 ROP GENE FUSION: POSITIVE SELECTION EXPRESSION VECTOR-ANTIGEN OVERPRODUCTION

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A plasmid vector has been constructed which has several features that facilitate high-level expression of foreign DNA in E. coli. These include: 1) a powerful, inducible promoter (Remaut et al., 1981), efficient translation initiation signals and 2) a positive genetic selection for recombinant plasmids carrying an insert. The vector, designated pMAM17, carries the ColE1 rop gene (Cesareni et al., 1982) under control of the thermally inducible λPL promoter. The rop gene product is a negative regulator of ColE1 DNA replication, and its high-level expression is lethal to cells. However, cells harboring a plasmid with an insert in the rop gene grow normally under these conditions. This provides a convenient method for the genetic selection and translational amplification of previously cloned DNA sequences. Our results demonstrate that induced extracts from cells harboring pMAM17 recombinants can produce 20-25% of their total soluble protein as rop hybrid protein. Moreover, unfractionated E. coli extracts containing rop-fusion protein can be used to obtain antibodies capable of specific recognition of authentic polypeptide present in cells from which the insert DNA originated (Muesing et al., 1984).

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A NEW HIGH-EFFICIENCY VECTOR FOR CLONING DNA IN *HAEMOPHILUS INFLUENZAE*

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RSF0885 is a multicopy plasmid of *Haemophilus* which carries an amp^r marker. Transformation with RSF0885 DNA of a highly competent *H. influenzae* culture for amp^r marker is surprisingly low. Absence in RSF0885 DNA of a specific 11-bp sequence thought to be required for uptake of any DNA into *H. influenzae* was considered to be an explanation for the low transformation efficiency. Splicing to RSF0885 of chromosomal DNA segments from *H. influenzae* genome which normally contains 600 copies of uptake sequences, improves the transformation 100-1000 fold. However, the chimeric plasmid DNA still requires *rec* gene expression for peak transformation.

RSF0885 is cut once with *Pvu*II and chromosomal DNA can be spliced to it and the inserts detected efficiently. A chimeric plasmid pD7 was isolated which carries a large segment of *H. influenzae* chromosomal DNA. pD7 has 4 *Eco*RI sites, all in the chromosomal segment. In two steps, a plasmid pJ1-8 was derived from pD7 which by itself gives little or no transformation and is cut only once with *Eco*RI. Its molecular weight is 2.5×10^6 versus 3.7×10^6 of RSF0885. Thus, pJ1-8 offers some advantages over RSF0885 such as (1) cutting can be done with an enzyme (*Eco*RI) that produces 'sticky' ends versus blunt-ends produced by *Pvu*II, (2) detection of colonies carrying inserts is more efficient since there are virtually no 'background' colonies and (3) a bigger insert can be put because of the plasmid being smaller now. Using this vector, two genes from *H. influenzae* have been cloned. Transformation with these chimeric plasmids suggests the involvement of recombination in DNA entry.

MOBILIZABLE VECTOR PLASMIDS FOR GENETIC MANIPULATIONS IN GRAM-NEGATIVE BACTERIA

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Genetic engineering vectors commonly used in *E.coli* (e.g. pBR325, pACYC184) have been modified to be applicable in a broad variety of gram-negative bacterial species (1,2).

There are two groups of plamids. Both are efficiently mobilizable in the presence of RP4 or other IncP plasmids, but they differ with respect to their replication host range.

One group, constructed by cloning the *oriT* region of RP4 into conventional *E.coli* vectors, can be introduced into all gram-negative species tested so far, but fails to replicate in bacteria outside the enterobacteria. This type of vector has for example been succesfully applied for general transposon mutagenesis and homogenotisation experiments in a variety of gram-negative strains.

The other group of mobilizable vector plasmids combines usual *E.coli* vector plasmids with the broad host range replication and mobilization functions of plasmid RSF1010. These vectors can therefore be introduced and also maintained in all gram-negative bacteria tested. They have been used for complementation experiments in *Rhizobium meliloti*.

We also constructed cosmid libraries of different Rhizobium strains with vector derivatives containing the λ *cos*-site. These experiments revealed that the replicative type of vector becomes unstable in *E.coli* and Rhizobium when carrying large inserts of foreign DNA. The cosmids are either completely lost (under non-selective conditions) or they retain their resistance marker (with selective pressure) but are degradated to plasmids of different sizes. This instability is markedly reduced in *E.coli recA* hosts.

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PROTEIN EXCRETION IN *ESCHERICHIA COLI*. APPLICATION OF THE PLASMID-ENCODED CLOACIN DF13 EXPORT SYSTEM

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The bacteriocin, cloacin DF13, excreted by the gram-negative bacterium *Escherichia coli*, has been studied extensively in our laboratory. Cloacin DF13 is encoded by the bacteriocinogenic plasmid Clo DF13. It consists of two subunits designated cloacin (m.w. 59,000) and immunity protein (m.w. 10,000). Both subunits are synthesized without a signal sequence and form an equimolar complex in the cytoplasm. Another pClo DF13 encoded polypeptide, designated protein H (m.w. 2,800), is responsible for the translocation of the bacteriocin molecules across the cytoplasmic and outer membrane.

The genes encoding the cloacin protein, the immunity protein and protein H are organized in one, mitomycin C inducible, operon with a common promotor in front of the cloacin gene. A model for the excretion mechanism of claocin DF13 will be presented. Furthermore the strategy for engineering protein excretion in *E. coli* by using this excretion system as well as preliminary results will be discussed.

NOVEL PLASMID-MEDIATED CATABOLIC PATHWAYS FOR HETEROCYCLIC COMPOUNDS

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A strain of Nocardia, capable of growing on pyridine-N-oxide (PNO), 2-hydroxypyridine and pyridine, catabolised PNO via 2-hydroxypyridine while pyridine was degraded via succinic semialdehyde; PNO and 2-hydroxypyridine did not serve as intermediates in this pathway. PNO-degrading ability was lost on cultivation in nonselective media, but such cells retained the ability to degrade pyridine and 2-hydroxypyridine. Pyridine-degrading ability of another Nocardia sp. growing on pyridine was also unstable, and lost spontaneously on storage or cultivation in non-selective media, suggesting the involvement of plasmids.

Two distinct pathways of quinoline catabolism have been deciphered employing four different organisms. A strain of Pseudomonas testosteroni and a Nocardia sp that arose as a contaminant in this culture, metabolised quinoline via 2-hydroxyquinoline and 2,6-dihydroxyquinoline. However, 8-hydroxycoumarin has been characterised as the key intermediate of quinoline catabolism in Pseudomonas stutzeri and another Pseudomonas sp.. These organisms metabolise quinoline via 2-hydroxyquinoline, 2,8-dihydroxyquinoline, 8-hydroxycoumarin and 2,3-dihydroxyphenylpropionic acid as intermediates. Coumarin was not utilised as a growth substrate by these organisms, but quinoline-grown resting cells converted coumarin to melilotic acid which was not further metabolised.

TRANSFER OF SHUTTLE PLASMID pTZ22 BY PROTOPLAST FUSION BETWEEN BACILLUS SUBTILIS Ki-2-132 AND ESCHERICHIA COLI C600

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Bacillus subtilis Ki-2-132 (Thr^-, Ile^-, Val^-) is a highly transformable mutant of Ki-2 which was screened from chinese soil. Its competence developed faster and stability of plasmids was higher than that of the commonly used strain 168. Plasmid pTZ22 is a shuttle plasmid between *B. subtilis* and *E. coli* and could be transferred by protoplast fusion from *E. coli* into *B. subtilis* and from *B. subtilis* into *E. coli*. The fusion frequency was 10^{-4}-10^{-6}. The secondary colonies, resembled *B. subtilis*, of fusion product colonies resembled *E. coli* were observed. The proteinase of *B. subtilis* Ki-2-132 did not seem to obstruct protoplast fusion, plasmid transfer, and gene expression in both directions of plasmid transfer.

RECOMBINANT PLASMIDS FOR THE ANALYSIS OF TRANSPOSITION

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Recombinant plasmids have been constructed carrying inverse and direct repeats of sites believed to be involved in recombination during transposition. These plasmids carry the RES site (TTATAA), identified as the site of action of the tnpR gene product, or a second site, termed "IRS", which contains a sequence of 23 bp, 19 of which are homologous with the 38 bp terminal inverse repeat of Tn2660. These two sites have been cloned separately, and jointly. (RES and IRS are respectively found at coordinates 2998 to 3003, and 3021 to 3043 of the Tn3 sequence, and Tn2660 appears to be identical in all respects to Tn3.

Rearrangements within the transposable element Tn2660 have been constructed to produce mutants in either the tnpA or the tnpR gene. Host cells carrying one of the mutant Tn2660 plasmids and a plasmid with an inverse or direct repeat of RES or IRS sites have been constructed in recA and $recA^+$ hosts and are being analyzed to determine if recombination events related to transposition occur. Deletions or inversions in the recombinant plasmids are expected as a consequence of the predicted activities of the tnpR and tnpA gene products. Identification of these events would further refine the current model of transposition.

HUMAN PROINSULIN EXPRESSION USING A COLICIN E3 DERIVATIVE VECTOR FOR THE DIRECT SELECTION OF RECOMBINANT DNA

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The screening of *E. coli* cells in cloning experiments can be done in a fast and efficient way by using a vector designed to allow a direct selection for recombinant plasmids. pVT25, a 3.94 kb plasmid, is a hybrid between the ampicillin resistance gene, the origin of replication of pBR327 and the colicin E3 gene (fig. 1). Since it does not carry the colicin E3 immunity gene such a plasmid becomes lethal for the host cells unless the bacteriocin is inactivated by insertion of a foreign DNA fragment. The number of recombinant clones obtained using pVT25 as a vector is proportional to the concentration of the DNA to be inserted. DNA fragments generated by ClaI, TagI, HpaII or AccI enzymes can be cloned into the single ClaI site of pVT25. This vector can be maintained within *E. coli* if complemented with another plasmid which expresses the colicin E3 immunity gene as well as the tetracycline phenotype.

The synthetic human proinsulin gene was fused in phase with the colicin gene of pVT25. Antigenic determinants against the C-peptide of proinsulin were shown to be expressed only after treatment of the cell culture with mitomycin C, a classic inducer of colicin synthesis. The maximum synthesis of the colicin-proinsulin hybrid protein was reached after three hours (fig.2).

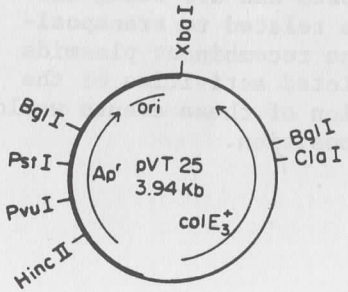

Fig.I

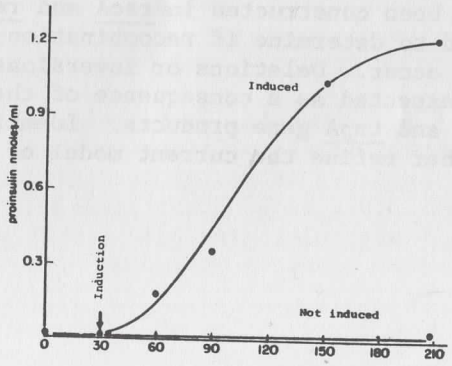

Fig.2

DEVELOPMENT OF SHUTTLE VECTORS BETWEEN STREPTOMYCES SPECIES AND *ESCHERICHIA COLI*

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Endogenous plasmids of *Streptomyces ghanaensis* and *Streptomyces venezuelae* were used to develop various shuttle vectors which replicate in Streptomyces as well as in *Escherichia coli*. The three Streptomyces plasmids which were characterized are suitable basic replicons for cloning experiments. They have a size between 12 and 14 kb, a copy number of about 20, and useful cloning sites for various restriction endonucleases. The shuttle vectors were obtained by fusing *E.coli* cloning vehicles like pBR325 and pACYC184 to the above described Streptomyces plasmids. In addition, antibiotic resistance genes for thiostrepton and neomycin (1) were cloned into these shuttle vectors. These resistance genes are expressed in a variety of Streptomyces strains. Further investigations in *S.lividans* 1326 revealed that these shuttle vectors can be transferred by conjugation between *S.lividans* strains. Transfer in *S.lividans* is accompanied by the typical pock structure described for *S.lividans* and *S.coelicolor* plasmids (2). The hybrids based on pACYC184 are stably maintained in *E.coli* and in the Streptomyces strains, so far investigated.

The shuttles are now used to study and to manipulate Streptomyces-DNA in *E.coli* and Streptomyces.

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THE ESCHERICHIA COLI INITIATOR PROTEIN, dnaA, IS INDISPENSABLE FOR THE MAINTENANCE OF λ PLASMIDS

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The plasmid pIG7 obtained by cloning the λ replication region (derived from the λdv1 plasmid) into the HindIII site of pBR322 transforms the polAts dnaAts Hfr cells with normal efficiency at 30°C. Due to polAts mutation, this hybrid replicon should be constrained to the λ-type of replication at 42°C although one cannot eliminate a possible cooperation of the origin of pBR322. At this temperature plasmid-less cells start to appear exponentially after a lag of about 2 hours, and the half-life in the loss of pIG7 is very close to one generation time. Hybridization of ^{3}H-thymidine pulse-labeled pIG7 DNA to the DNA of pBR322 points to the inhibition of replication of the hybrid plasmid at 42°C. The lack of dnaA activity may inhibit the replication of pIG7 directly, or indirectly - affecting the process of partitioning of the plasmid copies.

We have not been able to transform dnaAts Hfr cells with standard λ plasmids, even at 30°C. We obtained extremely rare transformants only for pRLM4 (1), using selection for kanamycin resistance. Plasmids isolated from these transformants revealed normal efficiency of transformation of dnaAts or dnaA^{+} cells at 30°C, and were shown to bear the $\widetilde{n}A$ type of mutation in the λ replication gene P (2, 3). When shifted to 42°C, the dnaAts Hfr bacteria harboring $\lambda\widetilde{n}$ plasmids start to segregate plasmid-less cells, as in the previously-described system. The results presented demonstrate the participation of functional dnaA protein in the maintenance of λ plasmids.

Since dnaA-independence of λ phage is well established, we postulate that λ phage may bypass the dnaA-dependent step (replication and/or partitioning) specific for λ plasmids.

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